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POLYPHENOLS IN CEREALS AND LEGUMES

Proceedings of a symposium held during the 36th annual meeting of the Institute of Food Technologists, St. Louis, Missouri, 10 – 13 June 1979

Editor: Joseph H. Hulse

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Introduction and Recommendations

Sorghum and the millets are the most important food crops of the semi-arid tropics (SAT), an ecological zone that almost circles the earth, beginning in China, continuing through India, and extending over most of Africa and parts of the southern United States. According to the FAO Production Yearbook 1977, the following are the approximate production areas in thousands of hectares under sorghum and millets: *Sorghum* — Africa 13 810, Asia 18 320, North and Central America 7460, South America 3260, World 43 650; *Millets* — Africa 16 360, Asia 45 730, South America 250, World 65 450.

Whereas in economically developed countries sorghum and millets are used mostly in animal feed (about 96% of total production versus 8% in developing countries), in the developing countries they are predominantly a source of food (about 83% of total production versus 1% in developed countries). About 3% of total production in developed countries goes to other uses including fermented beverages and industrial use versus 9% for such purposes in developing countries. It is probable that 90% of the rural people of the Sahelian zone rely upon sorghum and millets as their main source of food energy and together with legumes for most of their dietary protein.

Because of the importance of sorghum and millets in the diets of some of the world's poorest people, the nutritional quality of these grains and the diets of which they constitute a major component is a matter of serious concern.

During the past several years, the International Development Research Centre (IDRC) has undertaken a review of the composition and nutritive value of sorghum and those small-seeded cereal grains commonly known as the millets. The compilation of the results of this review is soon to be published (Hulse et al. 1980). One character of sorghum of particular interest and nutritional importance is the presence in the seeds of certain genotypes of significant levels of pigmented polyphenols. These polyphenols, when present, appear in the outer layers of the caryopsis, predominantly in the pericarp and/or testa. Their significance lies in their apparent biological activity: the high-polyphenol seed types reportedly being more immune to attack by birds and resistant to certain diseases and to preharvest germination. In addition, and more important, many high-polyphenol cultivars display an impaired nutritional quality when compared with low-polyphenol sorghum types of otherwise similar composition.

Much of the evidence of impaired nutritional quality derives from in vivo studies with laboratory and farm animals, and appears as a lower feed efficiency than nutrient analyses would suggest, diminished body weight gain per unit weight of feed intake, and reduced overall digestibility and protein utilization. On these effects the literature reveals a broad general agreement. The literature is less clear on the nature and mode of action of whatever sorghum polyphenols are responsible, the mechanism and cause of their formation, and whether other as yet unidentified substances are involved in the impaired nutritional quality.

Though most of the literature deals with sorghum, there is evidence that phenolic substances exist in the seeds of certain millets and some food legumes. All of these are crops of the semi-arid tropics and provide the main subsistence of some of the world's poorest peoples, therefore any impairment of their nutritional quality is of more than academic interest.

In spite of the general agreement that food grains high in polyphenols are nutritionally inferior to low-polyphenol types, the literature reveals many uncertainties, lacunae, and conflicting points of view. Consequently, it was decided to invite a group of scientists, all of whom have studied and published extensively on the subject, to review the state of relevant knowledge and to recommend what further research is necessary.

We are grateful to the President and Officers of the Institute of Food Technologists (IFT) for their permission to publish the proceedings and recommendations that emanated from the symposium and working group meeting.

It is hoped that this publication will be complementary to the Academic Press publication of the IDRC review and that the two will be studied together by research workers.

All of the authors of the following papers have made notable contributions to our knowledge of polyphenols in grains and each brought to the meeting important new information. Gupta and Haslam laid the basis for future research by prescribing a method of analysis by which was identified an important sorghum polyphenol. Butler, Price, and their colleagues at Purdue University have advanced our knowledge of the adverse biochemical effects of sorghum polyphenols and the means by which they may be neutralized or diminished. To Rooney and his colleagues at Texas A & M University we are indebted for much of what is known of the structure of the sorghum and millet grains and the principal sites of polyphenol concentration. Bullard has led the scientific field in relating polyphenol content to bird resistance. Bressani's research into the biochemistry of legumes is internationally recognized and his recent reports on nutritional differences between pigmented and light-coloured beans (*Phaseolus vulgaris*) are of unique importance. Reichert and his colleagues at Saskatoon, in addition to their identification of polyphenols in pearl millet, have made important advances in the technology of milling sorghum, pearl millets, and some food legumes. The value of their respective scientific contributions is amply illustrated by the contents of their respective papers.

Following the discussions, each member of the working group presented his recommendations for future research. These recommendations, edited to ensure a uniformity of presentation, are presented here.

Recommendations for Future Research

There appears to be a noticeable lack of agreement and precision in names given to those components of the grain that contain biologically active phenolics. (Some authors use "seed coat" when referring to the pericarp; and others variously name the mature ovule wall: seed coat, testa, subcoat, undercoat, inner integument, outer integument, or nucellar layer.) The phenolics themselves are variously described as phenolics, polyphenols, tannin(s), and tannin-like substances.

The formulation of a rational standard nomenclature for parts of the grain might be considered by the International Association of Cereal Chemists (IACC), to whom this publication will be sent for consideration.

Before the biologically active phenolic substances in the seeds of sorghum, various millets, and food legumes can be precisely named, much more research along the lines of that reported by Haslam and Gupta is needed to isolate and characterize the various phenolics produced in different genotypes at various stages of maturity. These substances can then be named according to the convention proposed by the International Union of Pure and Applied Chemistry (IUPAC) Commission on Nomenclature of Organic Chemistry.

In the meantime, it was recommended by the working group that as far as possible, the use of the word "tannin" be avoided and that all workers recognize there is no evidence of the presence in any of the grains of hydrolyzable tannins. This is emphasized because of the evidence of many instances in which "tannic acid" or "tannin" has been employed as a standard in the analysis of sorghum phenolics.

Though it appears that the modified vanillin hydrochloride (MVHCl) is probably the best method of analysis available, it was recommended that a more precisely standardized procedure be agreed upon by the working group for consideration by the IUPAC and IACC. The several sources of error inherent in the MVHCl method, and its dependence upon a reliable standard, were recognized by the working group. There is a need also for an in vitro biochemical method based upon either protein precipitation or enzyme inactivation. This will require close cooperation between chemists and biochemists — the former being required to isolate and purify the active compound(s) to enable the latter to prescribe standard methods of assay.

The working group did not propose that methods of biological evaluation, specific to the phenolics under consideration, be devised either for human or animal nutrition. It is proposed, however, to draw the general concern to the attention of the International Union of Nutritional Sciences, particularly the suggestion from one source that sorghum polyphenols may be carcinogenic. (Given the very tenuous and, indeed, doubtful evidence of carcinogenicity and the extreme excitement that the word "carcinogen" incites among the popular press, it must be emphasized that the working group sees no reason to discourage the consumption of sorghum on grounds of potential toxicity.)

The group recommends more research to elucidate the complex chemistry of polyphenolic-protein interactions. Several approaches will be called for including the influence of specificity and the degree of polymerization, and the thermodynamics of binding. A need was felt for a simpler method than the nuclear magnetic resonance (NMR) technique described by Haslam to determine the degree of polymerization. The "group II" sorghums reported by Bullard, the Purdue, and Texas A & M teams, deserve much greater study to determine if they contain a unique genetically controlled mechanism of bird resistance that does not embody adverse nutritional properties. This would appear, at first, a problem for the chemists to address.

As a general recommendation, it was proposed that all research to further clarify the chemical identity, location, concentration, mode of formation, and reactivity of the phenolics in grain be carried out on sorghums and other grains with clearly identified genetic characters and histories.

The state of knowledge concerning the identity of phenolics in the millets, important food legumes, and, in their dry form, pulses, is so sparse as to defy comprehensive recommendations. As a first step it seems desirable that the chemical structure of the pigments in dark-skinned *Phaseolus* beans be determined and that the studies of the apparent biological effects described by Bressani be continued. It was recommended that the methods of Haslam be applied to discover if there exists any structural similarity between the sorghum and bean pigments, respectively.

The valuable microscopic analyses reported by Rooney and his colleagues deserve to be continued and expanded to gain a better understanding of the sites of principal location and greatest concentration of the biologically active phenolics, and to determine differences in composition among the components of the caryopses of different genotypes at various stages of maturity, and how each of these differences is influenced genetically and by agronomic variation and environment.

There is a need to determine differences in composition, protein binding, and enzyme inactivation among phenolics from different sites in the caryopses of different genotypes.

Continued research is needed to determine the function and fate of the phenolics as the grain matures, and how they change in concentration and degree of polymerization. Of particular interest in these studies would be the difference in the pattern of behaviour between group II and group III sorghums, and in what manner significant differences are genetically controlled.

The need is evident to learn more of the relation between chemical composition and concentration of phenolics in food grains and their influence upon the nutritional quality of the host grain and other ingredients of human and animal diets. Complementary studies are necessary to determine in what manner the phenolic content influences acceptability of sorghum, the millets, and legumes to humans, farm animals, and avian creatures, and whether there are phenolics which, while acceptable and harmless to humans, are inhibitory to birds, insects, and other predators. Most important, it must be determined whether the phenolics present in sorghum caryopses are acutely or chronically toxic in any reasonable concentration.

Interrelated chemical and nutritional studies would be of particular importance in deciding in what manner the group II and group III sorghums are truly different.

Finally, because cereal grains and dried pulses are normally cooked or processed in some manner before being fed to humans or farm animals, an examination of the effect of traditional, conventional, and less conventional methods of processing on the phenolics and their nutritional effects is essential.

Several promising physical and chemical methods of reducing phenolic content are suggested in the following papers, but of particular concern are the reported findings at Purdue that in some of the sorghums high in phenolic content, the adverse nutritional effects are exacerbated during cooking. Our primary concern must be for the welfare of the poor people of the semi-arid tropics, therefore particular attention needs to be given to the influence on nutritional quality of traditional methods of cooking (see Vogel and Graham 1979) including long soaking (during which some fermentation and organic acid

formation occurs), the addition of tamarind (tartaric acid), and sour milk (lactic acid), and the various accepted processes of milling and fermentation.

Certainly a great deal of imaginative research is needed before we understand fully the implications of the phenolics present in sorghum, millets, and food legumes.

It was clearly not possible for all scientists directly interested in the subject to be present during the St. Louis symposium. It is hoped, however, that all who receive this publication will review the contents critically and send to us their comments and recommendations.

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Introduction et recommandations

Dans les tropiques semi-arides (SAT), cette zone écologique qui fait presque le tour de la terre, qui traverse la Chine, l'Inde, s'étend sur presque toute l'Afrique et réapparaît dans certaines régions méridionales des États-Unis, les cultures les plus importantes sont celles du sorgho et des divers genres de millet[1]. Selon l'annuaire de la FAO pour l'année 1977, les surfaces couvertes par les cultures étaient, en milliers d'hectares, les suivantes : *Sorgho* : Afrique, 13 810 — Asie, 18 320 — Amérique du Nord et Amérique centrale, 7 460 — Amérique du Sud, 3 260 — Total mondial, 43 650. *Millets* : Afrique, 16 360 — Asie, 45 730 — Amérique du Sud, 250 — Total mondial, 65 450.

Alors que dans les pays économiquement développés le sorgho et le millet sont principalement des nourritures animales (96%) et ne servent à l'alimentation humaine que dans une infime proportion (1%), ces chiffres sont inversés dans les pays en développement (8% et 83%). Environ 3% de la production, dans les pays développés, contre 9% dans les pays en développement, sert à d'autres utilisations comme la fabrication de boissons fermentées ou d'autres applications industrielles. Il est probable que 90% de la population rurale du Sahel a pour principale source d'énergie alimentaire le sorgho et le millet qui constituent, avec les légumineuses, la plus grande partie de son régime protéique.

Étant donné leur importance dans le régime de populations qui comptent parmi les plus pauvres du globe, il est justifié de se pencher sur la qualité nutritive de ces grains et des régimes dont ils sont un élément essentiel.

Ces dernières années, le Centre de recherches pour le développement international (CRDI) a entrepris une étude de la composition et de la valeur nutritive du sorgho et de ces céréales à petits grains que l'on groupe sous l'appellation commune de «millet». Les résultats de cette étude seront publiés prochainement par *Academic Press* (Hulse et al., 1980). Il existe chez le sorgho un caractère particulièrement intéressant par son importance nutritionnelle, à savoir la présence dans les grains de certains génotypes de quantités importantes de polyphénols pigmentés. Ces polyphénols, lorsqu'ils sont présents, peuvent être décelés dans les couches extérieures du caryopse, particulièrement dans le péricarpe ou le tégument. Leur importance viendrait de leur apparente activité biologique : les grains à forte teneur en polyphénols seraient plus immunisés que les autres contre les attaques des oiseaux, de même qu'ils résisteraient mieux à certains ravageurs et maladies. En outre, particularité plus importante encore, beaucoup de ces variétés à haute teneur en polyphénols sont inférieures sur le plan nutritionnel aux variétés à faible teneur, de structure semblable par ailleurs.

La preuve de cette particularité résulte en grande partie d'observations *in vivo* sur des animaux de laboratoire et de ferme, dont il ressort que le rendement alimentaire est inférieur à ce que l'analyse des substances nutritives laisserait supposer, que le ratio unité de gain de poids-unité alimentaire est en diminution,

1. Sauf exceptions, nous grouperons les «millets» ou «mils» sous le vocable courant de «millet».

comme le sont la digestibilité générale et l'utilisation physiologique des protéines. Les publications sur le sujet révèlent un large accord des observateurs, mais elles sont moins claires quant à la nature et au mode d'action de ces polyphénols du sorgho, au mécanisme et à la cause de leur formation et à l'action éventuelle d'autres substances non encore identifiées.

Bien que la plupart des publications ne parlent que du sorgho, il a été prouvé que les grains de certains millets et certaines légumineuses alimentaires renferment des substances phénoliques. Il s'agit justement de cultures des tropiques semi-arides, d'où tirent leur subsistance des populations parmi les plus déshéritées de la planète, de sorte que toute altération nutritive de ces aliments revêt un intérêt autre qu'académique.

Quoiqu'on s'accorde généralement pour admettre la qualité nutritive inférieure des céréales alimentaires riches en polyphénols, il existe dans les publications sur le sujet maintes incertitudes, lacunes et divergences. C'est pourquoi nous avons décidé d'inviter un groupe de scientifiques, connus pour leurs travaux et leurs publications sur le sujet, à faire le point des connaissances actuelles et proposer éventuellement des recherches plus poussées.

Nous exprimons notre reconnaissance aux président et membres du bureau de l'Institute of Food Technologists (IFT) pour nous avoir permis de publier les comptes rendus du Symposium et de la réunion du groupe d'études, ainsi que les recommandations qui en émanent.

Nous espérons que cette publication complètera l'étude du CRDI publiée par *Academic Press* et qu'en raison de leur caractère complémentaire elles seront etudiées ensemble par les chercheurs.

Tous les auteurs dont les noms suivent ont contribué substantiellement à l'étude de la teneur des grains en polyphénols et tous ont apporté au colloque des informations nouvelles et importantes. Gupta et Haslam ont jeté les bases d'une future recherche grâce à une méthode d'analyse qui a permis d'identifier un important polyphénol du sorgho. Butler, Price et leurs collègues de l'université Purdue ont avancé notre connaissance de la nocivité des effets biochimiques de ces polyphénols et des moyens à employer pour les neutraliser ou du moins les atténuer. Rooney et ses collègues de l'université du Texas A & M, quant à eux, nous ont appris la majeure partie de ce que nous savons de la composition des grains de sorgho et de millet, ainsi que de la localisation des concentrations de polyphénol. Bullard a pris les devants de la recherche en associant la présence de polyphénols à la résistance aux attaques des oiseaux. Bressani est connu mondialement pour sa recherche sur la biochimie des légumineuses, et ses récents travaux sur la différence nutritionnelle entre haricots pigmentés et haricots clairs (*Phaseolus vulgaris*) sont de toute première importance. Reichert et ses collègues de Saskatoon, outre qu'ils ont découvert des polyphénols dans le mil perlé (ou pénicillaire), ont fait avancer la technologie de la mouture du sorgho, des petits mils et de certaines légumineuses alimentaires. La valeur de leurs contributions est amplement illustrée par le contenu de leurs communications scientifiques.

Après les discussions, chaque participant a fait des propositions en vue des futurs travaux de recherche. Nous allons les reproduire ci-dessous, sous une présentation uniformisée.

Propositions en vue des futurs travaux de recherche

Il semble qu'il y ait une absence apparente d'assentiment et de précision dans la désignation des parties du grain qui contiennent des phénols biologiquement actifs. Certains auteurs parlent de «l'enveloppe du grain», pour le péricarpe, et d'autres emploient des noms variés pour ce que nous désignerions comme le «tégument». Même hésitation en ce qui concerne les phénols : phénols, polyphénols, tanins, quasi-tanins, etc. Nous allons donc envoyer cette publication à l'International Union of Cereal Chemists (IACC) pour normalisation éventuelle de la nomenclature.

Avant que les substances phénoliques biologiquement actives présentes dans les grains de sorgho, du millet et dans les légumineuses alimentaires puissent être nommées avec précision, il conviendrait de se livrer à plus de recherche sur la base des indications de Haslam et Gupta, afin d'isoler et de caractériser les divers phénols produits dans divers génotypes à divers stades de maturité. Ces substances pourraient alors être désignées selon la convention proposée par la Commission sur la nomenclature de la chimie organique de l'International Union of Pure and Applied Chemistry (IUPAC).

Entre-temps, le groupe d'études recommande que, dans la mesure du possible, le mot «tanin» soit évité et que tous les chercheurs reconnaissent qu'il n'existe aucune preuve de la présence dans les grains de tanins hydrolysables. Si ce point a été fortement souligné, c'est qu'il y a eu beaucoup de cas où les mots «tanin» et «acide tannique» ont été employés comme normatifs dans l'analyse des phénols du sorgho.

Bien que la meilleure méthode d'analyse connue soit, apparemment, l'emploi d'hydrochlorure de vanilline (MVHCl), nous avons recommandé que le groupe d'études se prononce en faveur d'une procédure normalisée plus rigoureuse qui pourrait être adoptée par l'IUPAC et l'IACC. Le groupe d'études a reconnu que la méthode MVHCl est sujette à des erreurs de différentes sources et dépend d'une norme fiable. La nécessité se fait sentir également d'une méthode biochimique appliquée *in vitro*, basée soit sur la précipitation de protéines, soit sur l'action d'un anti-enzyme. Cela suppose une étroite collaboration entre chimistes et biochimistes, les premiers devant isoler et purifier les composés actifs afin que les seconds puissent préconiser des méthodes normalisées.

Le groupe d'études n'a pas proposé d'établir des méthodes d'évaluation biologique applicables spécifiquement aux composés phénoliques en cause, pour la nutrition tant humaine qu'animale. Il préconise par contre de porter à l'attention de l'Union internationale des sciences de la nutrition toutes ces questions et particulièrement celle de la possibilité que les polyphénols soient cancérigènes, comme on l'a suggéré. Disons tout de suite qu'en raison du peu de vraisemblance et de l'absence presque totale de preuve de cette hypothèse comme de la résonance extrême du mot «cancérigène» dans la presse populaire, le groupe d'études ne voit aucune raison de décourager la consommation du sorgho au motif d'une éventuelle toxicité.

Le groupe recommande plus de recherche afin d'élucider la complexité chimique des interactions polyphénoliques et protéiques. On devra faire appel à plusieurs approches, impliquant notamment l'influence de la spécificité, le dégré de polymérisation et la thermodynamique de la liaison. Il faudrait apparemment recourir à une technique plus simple que celle de la NMR (nuclear magnetic

resonance) décrite par Haslaın, qui sert à la détermination du degré de polymérisation. Les sorghos du «groupe II» dont parlent Bullard et les équipes des universités Purdue et Texas A & M méritent une étude plus attentive. Il faudrait déterminer s'ils contiennent un mécanisme particulier à commande génétique qui organiserait une résistance aux attaques des oiseaux sans entraîner de dommages aux qualités nutritives. Il semble qu'à première vue, ce soit là un problème à résoudre par les chimistes.

D'une façon très générale, il a été préconisé que toute recherche visant à déterminer la composition chimique, le point de fixation, la concentration, le mode de formation et la réactivité des phénols dans les grains de sorgho ou autres mette clairement en évidence les caractères génétiques et les antécédents de ces céréales.

Nos connaissances sur la composition des phénols dans le millet et d'importantes légumineuses alimentaires, fraîches et sèches, sont si éparses qu'il est impossible de formuler des recommandations complètes. Pour commencer, il serait désirable de déterminer la composition chimique des pigments du *Phaseolus* à tégument foncé, de poursuivre les travaux de Bressani sur les effets biologiques visibles de la pigmentation. L'application des méthodes de Haslam a été préconisée: il s'agit de savoir s'il existe une similitude de composition entre les pigments du sorgho et ceux du haricot.

Les analyses microscopiques de Rooney et de ses collègues sont intéressantes; elles méritent d'être continuées et étendues afin de permettre une meilleure compréhension des points de principale fixation et concentration des phénols biologiquement actifs, et de permettre également de déterminer les différences de structure des composants des caryopses de divers génotypes à divers stades de leur maturité et comment ces différences proviennent d'influences génétiques, agronomiques et environnementales.

Il faudrait déterminer les différences de composition, de liaison des protéines et de neutralisation des enzymes entre les phénols relevés à divers points de fixation dans les caryopses de divers génotypes.

Il faudrait aller plus loin dans la recherche pour découvrir la fonction et la finalité des phénols à mesure de la maturation des grains, de même que les voies de changement de leur concentration et leur degré de polymérisation. Il serait particulièrement intéressant d'étudier la différence de comportement entre les sorghos du groupe II et ceux du groupe III et de quelle façon les différences importantes sont commandées génétiquement.

A l'évidence, il faudrait en savoir davantage sur la relation entre la composition chimique et la concentration des phénols dans les céréales alimentaires et leur influence sur la qualité nutritive des grains-hôtes et les autres ingrédients des régimes de l'homme et des animaux. D'autres études devront déterminer de quelle manière la présence de phénols dans le sorgho, le millet et les légumineuses peut les rendre impropres à la consommation humaine, à celle du bétail et de la volaille, à celle des créatures ailées et s'il existe des phénols qui, bien que propres à la consommation humaine et inoffensifs pour l'homme, ont un effet dissuasif sur les oiseaux, insectes et autres ravageurs. Chose plus importante encore, il conviendrait de savoir si les phénols, lorsqu'ils existent en concentration tant soit peu importante dans le caryopse du sorgho, présentent une toxicité quelconque de caractère aigu ou chronique.

Pour décider de quelle façon diffèrent vraiment les sorghos des groupes II et III, il faudrait se livrer à des études portant à la fois sur la chimie et la nutrition.

Finalement, étant donné que les grains des céréales et les légumineuses sèches subissent habituellement une cuisson ou autre préparation avant d'être consommés par l'homme ou l'animal de ferme, il est essentiel que l'on étudie les effets des méthodes de traitement classiques et d'autres moins traditionnelles sur les phénols et leurs effets nutritionnels.

On lira dans les articles qui suivent des propositions intéressantes de réduction du contenu phénolique par des procédés chimiques et physiques, mais on trouvera sans doute du plus grand intérêt les constatations des chercheurs de l'université Purdue sur la maximisation de la nocivité nutritionnelle, pendant la cuisson, de certains sorghos à forte teneur en phénol. Notre premier souci doit être celui du bien-être des populations déshéritées des tropiques semi-arides; il convient donc de s'attacher particulièrement aux conséquences sur leur nutrition des méthodes culinaires traditionnelles (voir Graham et Vogel, 1979), notamment de la macération des aliments (qui les fermente et provoque la formation d'acides organiques), de l'addition d'acide tartarique (du fruit du tamarinier) et d'acide lactique (de lait tourné) ainsi que de divers procédés de mouture et de fermentation.

Il faudra certainement encore beaucoup de recherche imaginative avant que nous puissions bien comprendre les conséquences de la présence de phénols dans le sorgho, les millets et les légumineuses.

Il était évidemment impossible à tous les scientifiques directement intéressés d'assister au Symposium de Saint-Louis. Nous espérons toutefois que tous les destinataires du présent document en feront l'étude critique et enverront commentaires et propositions à :

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Vegetable Tannins — Structure and Biosynthesis

R. K. Gupta[1] and E. Haslam[1]

One of the most distinctive groups of higher plant secondary metabolites is the vegetable tannins. These are polyphenols (molecular weight 500–3000) whose ability to complex and precipitate proteins is germane not only to considerations of higher plant metabolism but also to numerous aspects of food technology. Their structure, biosynthesis, and interaction with proteins will be reviewed with particular reference to the tannin — a procyanidin — elaborated in the seed coat of sorghum.

Evolution and function lie almost synonymously at the heart of biology, and with the flowering of biochemistry in the twentieth century it was apparent that to many natural products, isolated by chemists in the eighteenth and nineteenth centuries, a distinctive role in the life of organisms could now be assigned. Thus citric acid and L-malic acid are essential to carbohydrate metabolism as key intermediates of the ubiquitous tricarboxylic acid cycle; adenosine is a constituent of nucleic acids; the various α-amino acids are the building units of proteins; fatty acids function as integral parts of complex lipid structures; and lanosterol (isolated from sheep's wool) is a key intermediate in steroid biosynthesis. These substances are classified as primary metabolites and, moreover, they are found in broadly similar patterns in most, if not all, living organisms. In contrast, there was an infinitely greater body of substances, each of which had a sporadic distribution in living matter, and to which no specific function could be assigned. Attention was first drawn to this group by plant physiologists, and they are now commonly referred to as secondary metabolites. As Bu'Lock (1965) has remarked, they express the individuality of a species in chemical terms.

While ignorance of their biochemical function and significance prevails at the experimental level, not surprisingly, theories and speculation abound at the philosophical level.

Opposing viewpoints have developed. One theory propounds that they are waste products or "accidents of metabolism" (Muller 1969), notwithstanding the fact that many are toxic to the plant or microorganism unless dissipated into the environment (e.g. volatile monoterpenes), or are harmlessly sequestered in the plant itself (e.g. phenolic glycosides). On the other hand, a contrary opinion maintains that these substances possess (or possessed) pertinent biological functions (e.g. Fraenkel 1959), and the example of the vegetable tannins is frequently cited in support of this view. The importance of vegetable tannins to the plant lies, it is believed, in their effectiveness as repellents to predators whether animal or microbial. According to Bate-Smith (1954), the relevant property is *astringency*, which for animals renders the plant tissue unpalatable by precipitation of salivary proteins and for parasitic organisms impedes the invasion of the plant tissue by immobilizing extracellular enzymes. It is persuasively argued that this strong association with proteinaceous materials is a primary function that has been of considerable evolutionary significance in the plant kingdom.

After the early encouragement that emerged from Emil Fischer's outstanding contributions to the chemistry of vegetable tannins (Fischer 1919), chemists were slow to recognize the complexity of the problems these substances posed, and over the next 40–50 years it became one of the untidy corners of organic chemistry. Nonetheless, the recently obtained knowledge of the structure, molecular shape, biosynthesis, and chemical reactivity of the principal vegetable tannins (Haslam 1966 and 1977; Mayer 1973), now makes possible, for the first time, a systematic examination of their biochemical and biological properties. This must surely be the aim of future work.

It is not possible to give a concise definition of the word "tannin" and the inability to do so has led to numerous misunderstandings in the literature. Tanning is a process whereby an animal skin is turned into leather, and its essence is to bring about cross-linking of the collagen chains in the skin and thus to protect the protein fibres from microbial attack and give the skin greater resis-

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tance to water, heat, and abrasion. During this process, which uses vegetable extracts, the skin may adsorb up to half its weight of "tannins." In this sense the implication of the word "tannin," and indeed its original use by Seguin (1796), clearly indicates a substance that produces leather from hide. In plant extracts these substances are polyphenols of varying molecular size and complexity. Invariably they constitute only a limited proportion of the total polyphenols in a plant tissue, and the failure to make this critical distinction has led one writer (White 1957) to suggest that "much of the botanical data concerning the occurrence of tannins in plants is of doubtful validity since it is based on tests which are insufficiently specific." In a nutshell, the general criteria for phenols (colour tests etc.) are quite inadequate to determine the presence of vegetable tannins.

Bate-Smith and Swain (1962) have adopted the earlier ideas of White (1957) to formulate a definition of vegetable tannins that, with present knowledge, is the most useful one to follow. These authors defined vegetable tannins as "water-soluble phenolic compounds having molecular weights between 500 and 3,000 and, besides giving the usual phenolic reactions, they have special properties such as the ability to precipitate alkaloids, gelatin and other proteins." This is the definition used here with the added proviso that the phenols are normal metabolic products and are not in vitro transformation products formed by chemical or other means. Nonetheless, it is well to note that this definition derives from considerations of the ways in which polyphenols tan protein fibres of animal skins and as such it groups together a series of phenolic compounds that possess this common characteristic. Clearly this property may be a purely fortuitous one and from the point of view of plant metabolism and plant biochemistry as a whole it may, in the final analysis, be a quite misleading one.

The ability to complex with proteins makes vegetable tannins distinctive metabolic products because formation of substantial quantities of free tannin molecules within the cytoplasm would presumably cause precipitation of structural and catalytic proteins. In this respect they resemble several antibiotics that are more toxic to the producing organism than are their precursors. The presence of vegetable tannins in plant tissues nevertheless has several important practical consequences. The interaction of the tannins with the salivary proteins and glycoproteins in the mouth renders the tissue astringent to the taste and this characteristic may determine one's enjoyment of particular fruit (e.g. blackberry, strawberry, cranberry, and apple). Firmly established in the biochemical literature (Howes 1953; Goldstein and Swain 1963) is the belief that changes in the palatability of many fruits that occur on ripening are associated with concomitant changes in the concentration of tannins present in the fruit. A widely expressed view is that the astringency of green immature fruit is due to the presence of tannins but that on ripening these are much reduced in quantity or are modified in some unspecified way.

Structure of Vegetable Tannins

The most acceptable major division of vegetable tannins is one first suggested by Freudenberg (1920) and is based on structural types. It separates the tannins into two classes, the hydrolyzable and the nonhydrolyzable or condensed. Simple treatment of hydrolyzable tannins with acid or alkali and in certain cases hydrolytic enzymes (tannase) splits them into sugars and some recognizable phenolic carboxylic acid (e.g. gallic acid or ellagic acid). Condensed tannins do not readily break down in this way, nor do sugars contribute to their overall structure. From the point of view of the association with proteins and other natural macromolecules, the two classes simply illustrate the principal means whereby plants can elaborate polyphenolic molecules with sufficient phenolic groups to form multiple hydrogen bonds with the substrate. Typical examples are Chinese gallotannin (syn. tannic acid, galls-*Rhus semialata*) and the condensed procyanidin that forms the major polyphenol from sorghum grain.

The histological reaction for vegetable tannins in plants is most commonly due to the presence of proanthocyanidins (referred to in earlier papers as leucoanthocyanidins and synonymous with condensed tannins), and they are present, often in substantial quantities, in many dicotyledons and in the most primitive of vascular plants such as ferns and gymnosperms. Their appearance in plants appears to be associated phylogenetically with the ability to form lignified tissues and with the development of a vascular character. Of the various proanthocyanidins those which form cyanidin on acid treatment — the *procyanidins* — are most widely distributed in plants. Invariably they co-occur alongside one or both of the flavan-3-ols: (+)-catechin or (-)-epicatechin.

Ideas on the biosynthesis of the plant procyanidins and associated flavan-3-ols are based on a range of structural observations and biosynthetic experiments (Haslam 1977). The results of these experiments are summarized in Fig. 1 in a path-

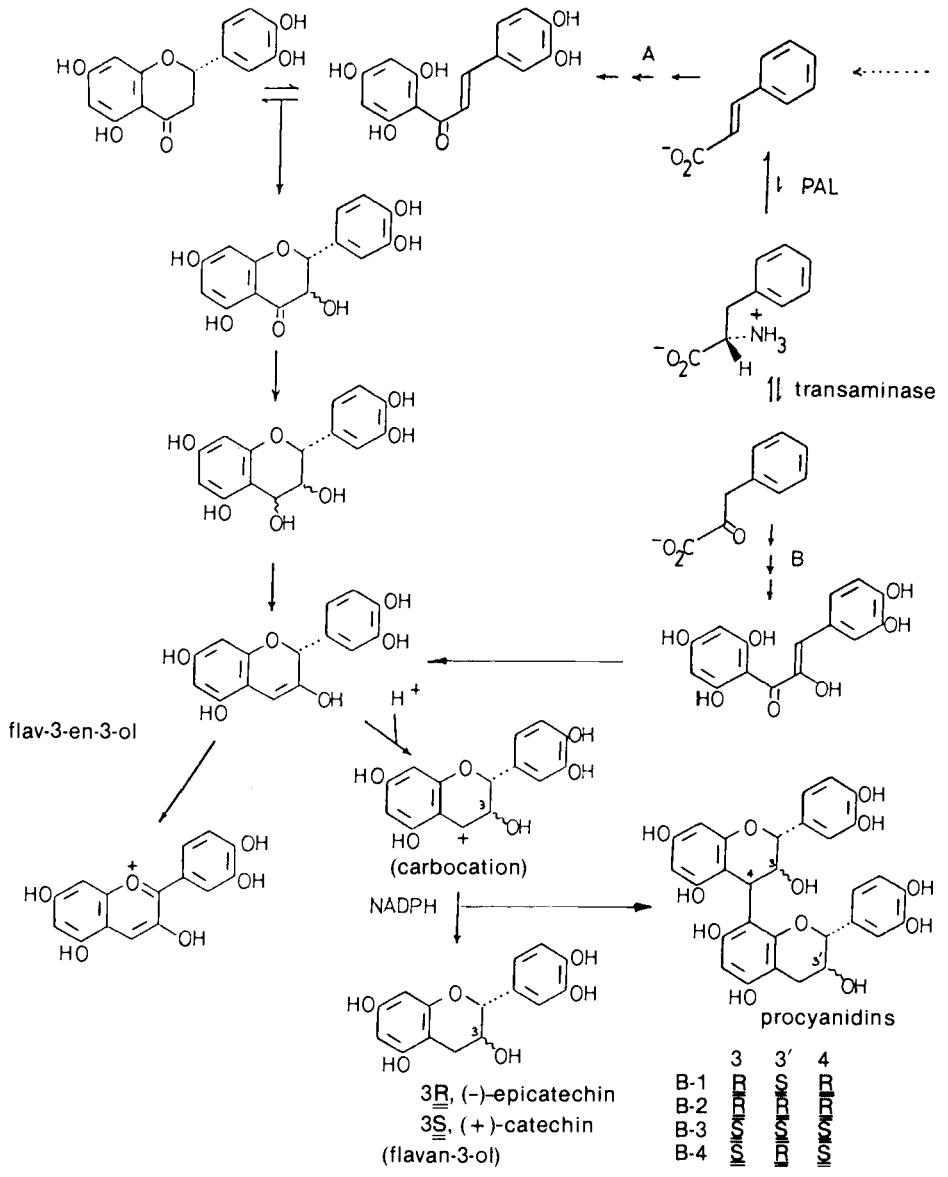

Fig. 1. Biosynthesis of procyanidins.

way of procyanidin metabolism that accords in broad outline with the tenets of flavonoid biosynthesis outlined by previous workers, most notably Grisebach (1979) — namely that a $C_6 \cdot C_3$ fragment (cinnamate) is combined with three acetate-derived units to form the $C_6 \cdot C_3 \cdot C_6$ carbon skeleton of the flavonoids. The chalcone ⇋ flavanone pair is the first formed intermediate, but the sequence of chemical changes in the C_3 unit that results in the formation of the individual flavonoids themselves remains poorly defined. In Fig. 1 a plausible scheme from the chalcone ⇋ flavanone pair to the flavan-3-ols [(+)-catechin and (-)-epicatechin] is shown. A key intermediate is believed to be the flav-3-en-3-ol and a two-step sequence (stereospecific protonation followed by reduction) is postulated to yield the flavan-3-ols.

The procyanidins are thought to arise as by-products in this two-stage process when the

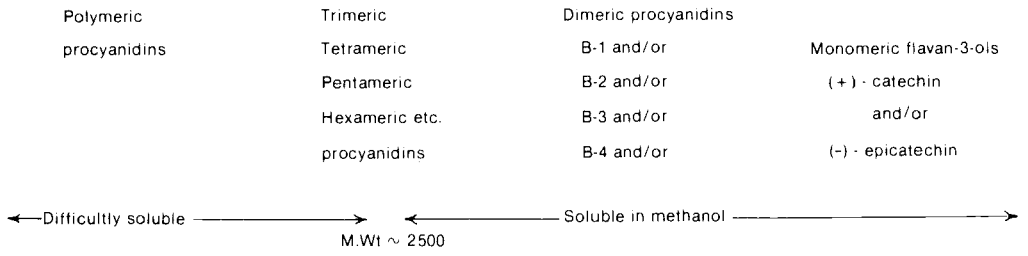

Fig. 2. Procyanidins in plant tissues.

supply of biological reductant (NADPH) is rate limiting. The various distinctive patterns of procyanidins found in plants are then thought to arise by reaction of one or both of the intermediate carbocations that escape from the active site of the enzyme with one or both of the flavan-3-ols utilizing their nucleophilic reactivity at carbon atom C-6 or C-8. The dimeric forms are first produced, but these react similarly with further carbocations to produce trimers, tetramers, and higher oligomers. Thus any plant produces a range of procyanidins of differing molecular weight as shown in Fig. 2. Only those flavan-3-ols with molecular weights up to about 3000 are soluble, and it is not known whether those of higher molecular weight are simply insoluble or are in fact attached in some way to skeletal tissues of the plant. It should, however, be emphasized that these difficultly soluble materials frequently represent the major fraction of procyanidin materials in a plant tissue. The question of whether polyphenol metabolism of this type continues over a long period with turnover of the products is somewhat contentious, but our belief is that it does not. Our view is that there is a relatively short "burst" of procyanidin synthesis after which the level of product remains approximately the same in the plant tissue until senescence occurs.

The soluble monomeric, dimeric etc. procyanidins thus represent only the "tip of the iceberg" for the range of procyanidins found in any given plant species. Two-dimensional paper chromatography of these soluble forms does, however, give readily recognizable patterns (fingerprints) that permit plants to be readily categorized (Fig. 3). Thus, for example, *Malus* sp., *Prunus* sp., and *Crataegus* sp. metabolize (-)-epicatechin, procyanidin B-2 etc.; *Salix* sp. and *Fragaria* × *ananasa* produce (+)-catechin, procyanidin B-3 etc. The most characteristic reaction of procyanidins is their acid-catalyzed degradation to give the pigment cyanidin by rupture of the interflavan bond, and this degradation proceeds by initial formation of the appropriate carbocation from the "upper half" of the dimeric procyanidin (Fig. 4). The carbocation is normally converted by proton loss and oxidation to give cyanidin, but it may also be intercepted under appropriate conditions to give flavanyl-4-thioethers, and this reaction has proved to be of great importance in subsequent identifications.

Sorghum Procyanidins

"There are no applied Sciences . . . there are only applications of Science and this is a very different matter . . . . The study of the application of Science is very easy to anyone who is master of the theory of it."

Louis Pasteur

Sorghum is an important food grain whose nutritional quality is considerably diminished in many hybrids by the presence of "tannins." Methanolic extracts of "very high-tannin" sorghums were analyzed initially by paper chromatography and these immediately showed one of the characteristic procyanidin "fingerprints" noted above. In particular the flavan-3-ol (+)-catechin and the procyanidin dimer B-1 were readily identified. Subsequently, large-scale extraction of sorghum cultivars gave extracts in which only polymeric procyanidins were present and in which the dimer B-1 and (+)-catechin were detected in only trace amounts. Later work revealed some of the possible reasons for this apparent change in the spectrum of phenols present in different cultivars. The sorghum grain is formed initially in a sheath, and at the etiolated stage no procyanidins can be detected. As chlorophyll develops in the seed coat there is an apparently rapid synthesis of polyphenols and both B-1 and (+)-catechin are present. However, as the seed ripens (to a red-brown appearance) these monomeric and dimeric flavan-3-ol species appear to decline rapidly in concentration to leave the polymeric procyanidin as the principal and in many cases the sole procyanidin in the seed coat.

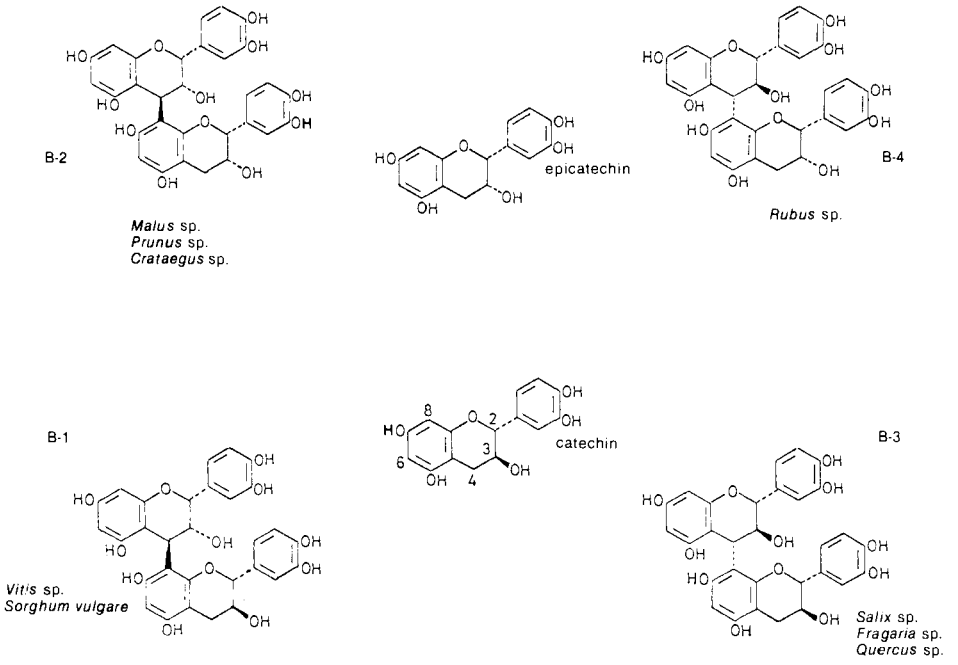

Fig. 3. Procyanidins: natural sources.

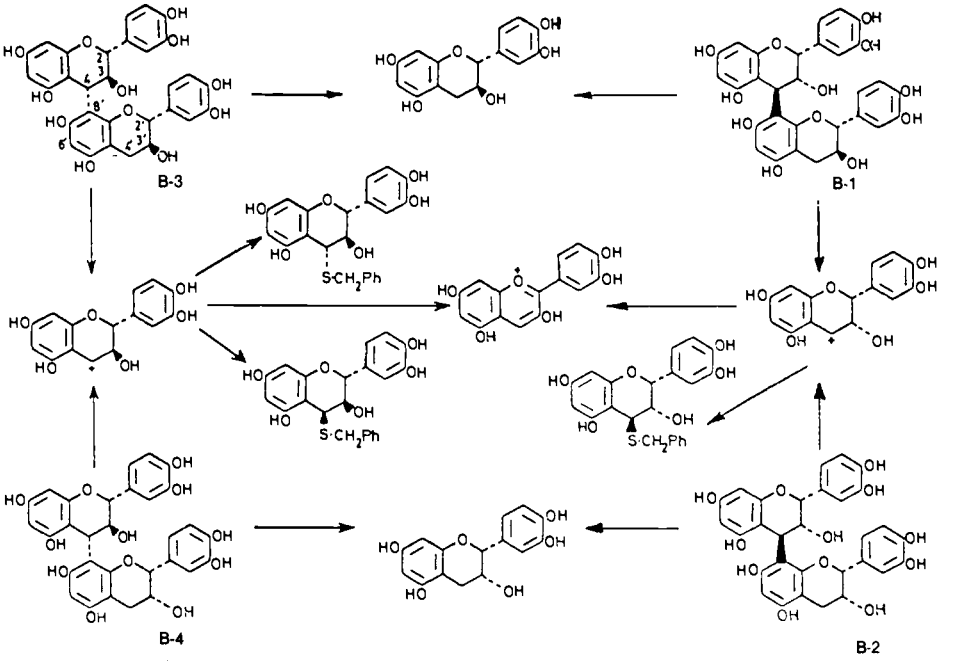

Fig. 4. Procyanidin dimers: degradation.

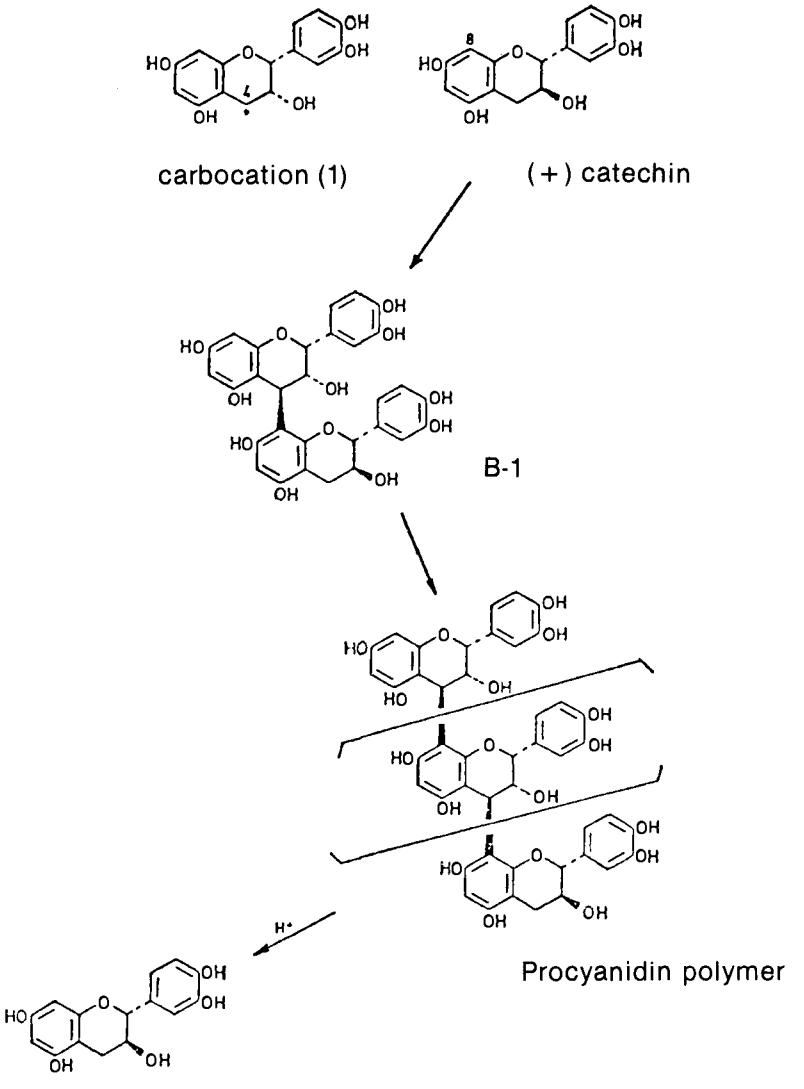

Fig. 5. Structure and biosynthesis of procyanidin polymer from Sorghum NK 300.

The polymeric procyanidin (up to ca. 5% of the grain) was isolated after chromatography of sorghum extracts on Sephadex LH-20 and gave analytical figures corresponding to a polytetrahydroxyflavan-3-ol structure. It contained, when pure, no sulfur or nitrogen. Treatment of the polymer with hydrochloric acid in ethanol at 60 °C gave cyanidin; it was therefore subsequently degraded by two acid-catalyzed procedures.

In this way the procyanidin polymer — which is the principal if not sole vegetable tannin of sorghum — was characterized as a hexameric-heptameric polyflavan-3-ol (Fig. 5, n = 4 or 5) with an average molecular weight of 1700–2000 (Haslam and Gupta 1978). On the basis of previous work, and the observations outlined earlier, the polymeric procyanidin in sorghum cultivars may be presumed to be formed from (+)-catechin and the carbocation (1) in a multiple condensation process to give a polymeric structure of the general form shown in Fig. 5 in which the interflavan bonds are formed predominantly but not exclusively between C-4 and C-8 of the various flavan units.

Analysis of Procyanidins in Sorghum

The Vanillin Reaction

The method of quantitative analysis for tannins that has become most widely used for sorghum grain in the laboratory is that of the reaction of the polyphenols present with vanillin and hydrochloric acid. This reaction is not specific for polyphenols that are tannins and the reagent will react with any phenol that has an unsubstituted resorcinol or phloroglucinol nucleus activated toward electrophilic substitution in the molecule. It is therefore specific for a very narrow range of flavan-3-ols, dihydrochalcones, and proanthocyanidins, but it will not discriminate between these on the basis of molecular weight. In the sorghum analysis it will measure the total of flavan-3-ols and higher oligomers (which include the tannins).

The procedures outlined in the literature for the vanillin assay do not, in some cases, adequately define the conditions employed and the parameters that may be varied. Several features of the reaction and the method were therefore examined: these included the concentration of vanillin and hydrochloric acid in the reagent, the temperature at which the reaction was conducted, the choice of appropriate standards for the reaction, and various extraction procedures. The following conditions were found to be most suitable:

(1) Sorghum grain was ground to a fine powder (to pass a 0.5 mm sieve) and a sample (5.0 g) extracted with methanol (total volume 200 ml, 4 × 50 ml) in a high-speed mixer (10 min each extraction). The methanol extracts were filtered free and combined and reduced to a small volume at 30 °C by rotary evaporation. The extract was then transferred to a graduated flask with methanol and the final solution made up to 50 ml with methanol.

(2) The reagent was prepared by mixing *just prior* to use equal volumes of a vanillin solution (4% in methanol) and hydrochloric acid (8% of 12*N* acid in methanol). All solutions must be prepared as required.

(3) Aliquots of the sorghum grain extract (1.0, 2.0, 3.0, 4.0, and 5.0 ml) were added to graduated flasks and the volumes made up as required to 5.0 ml with additional methanol. From each flask an aliquot (1.0 ml) was removed and added to the reagent solution (5 ml) in a small cuvette at 30 °C and thoroughly mixed. After 30 min the optical density of the solution was measured at 500 nm using the reagent in the blank cell. Each measurement was made in triplicate as described above and a graph of optical density versus concentration constructed.

(4) Sorghum procyanidin polymer (NK 300, 25 mg — extracted as previously described) was dissolved in methanol (50 ml) and aliquots (1.0, 2.0, 3.0, 4.0, and 5.0 ml) taken as above and added to flasks and made up to 5.0 ml in volume. Aliquots (1.0 ml) were taken from each solution and reacted with the reagent solution (5 ml) for 30 min at 30 °C. The optical density of the solutions was measured at 500 nm and a calibration curve constructed from measurements made in triplicate. Comparison of the slopes of the standard and unknown graphs gave the concentration of tannin in the extract.

(5) Some comments on this method are appropriate. The vanillin reaction is a temperature dependent one and for accuracy and reproducibility it was found that the reaction must be conducted at a fixed temperature. Variation in the concentration of vanillin and of concentrated hydrochloric acid also were discovered to cause fairly wide variations (20–30%) in the observed absorbance at 500 nm for catechin, epicatechin, procyanidin dimers B-2 and B-3, and for the polymeric procyanidin (tannin) isolated from Sorghum NK 300. Most surprising, perhaps, has been the observation that the time courses for colour development for the various substrates in the vanillin assay are quite different and it has not yet been possible to decide precisely what the reasons are for these differences. It is clear that the reaction kinetics are markedly different for different substrates and that the reactions occurring in the assay are rather more complex than previously believed.

These observations are broadly responsible for the choice of reaction conditions in the vanillin assay and for the choice of the sorghum procyanidin polymer as standard for the assay. Given these restrictions the vanillin assay appears to be a workable and reproducible procedure. However, it must be borne in mind that the results from this method are generally likely to be high relative to other methods, because the reaction measures low as well as high molecular weight phenols and therefore does not measure tannins alone. Alternative procedures have therefore been examined in an attempt to devise methods more specific for the tannins in sorghum.

Ultraviolet Absorption

In contrast to low molecular weight phenols, the polyphenols of sorghum that constitute the tannin fraction are absorbed from ethanol solution onto Sephadex LH-20 and are only removed by elution with methanol. This observation forms the basis of the second procedure utilized to analyze the tannins in sorghum. The general pro-

cedure is outlined below. It is important that all the solvents used should be redistilled.

An extract of the polyphenols in the sorghum grain (5.0 g) was prepared in methanol (50 ml) as described above in the vanillin assay. An aliquot of this solution (2.0 ml) was applied to a column of Sephadex LH-20 in ethanol (25 × 2.5 cm). The methanol solution was absorbed onto the column and the column was then eluted with ethanol (500 ml) to remove low molecular weight polyphenols. The residue of soluble phenolic materials still retained on the column consists principally of the polymeric procyanidin and this may be eluted with methanol (1000 ml). The methanol eluate was concentrated at 30 °C and finally made up to a volume of 100 ml in a graduated flask. The optical density of this solution was measured at 280 nm and the amount of tannin determined from a standard concentration versus optical density graph prepared from authentic polymeric procyanidin (NK 300). Each analysis was carried out in triplicate and the mean of three values obtained.

This method is simple to operate, but it is also extremely tedious and time consuming, and this is its great disadvantage. Thus, whereas the vanillin assay may permit several samples of grain to be analyzed on the same day, one assay of a sorghum grain (carried out in triplicate) using the ultraviolet absorption method takes from 3 to 4 days to complete. This is due to the very slow elution rate of Sephadex LH-20 columns. The method is also an expensive one in terms of the utilization of solvents and Sephadex LH-20. It is however, we believe, relatively specific for the tannins in sorghum.

The Cyanidin Coloration Method

This procedure makes use of the fundamental characteristic of procyanidins — that is the formation of the red pigment cyanidin when heated with acid. Using this procedure all procyanidins (dimers, trimers, and high oligomers) react and are thus estimated by the chromogenic reaction. Whether dimers and trimers are tannins is in itself questionable, but from our own observations their concentration is very low in all the sorghum samples that we have examined. The procedure is briefly outlined below.

In this method a methanol extract (50 ml) of sorghum seeds (5 g) was prepared as outlined earlier (vanillin assay, above). Aliquots (0.5 ml, 1.0, 1.5, and 2.0 ml) were taken, added to conical flasks (50 ml), and evaporated to dryness. Butan-1-ol (10 ml) containing hydrochloric acid (12*N*, 30% v/v) was added to each flask and the solutions heated at 115 °C for 2.5 h. After cooling, the solutions were transferred to graduated flasks and the volumes made up to 25 ml with butan-1-ol. The optical density of each solution was measured at 545 nm, and a graph was plotted of optical density versus concentration. Comparison with a standard calibration graph obtained analogously using the polymeric procyanidin from Sorghum NK 300 then gave the percentage of tannin in the unknown.

Some comments on this procedure are important. The development of cyanidin pigmentation from procyanidins by heating in alcoholic mineral acid is not a straightforward reaction. Side reactions occur with the formation of other coloured products (phlobaphens) and it is important that those interfere as little as possible with the colorimetric estimation. Maximum colour development is dependent both on the temperature at which the reaction is conducted and upon the acid strength of the media. Fairly wide differences (10–50%) were observed upon changing the acid (HCl) content of the reaction medium (10–60%). Some pigment degradation may also occur after long reaction times (> 3 h) but maximum colour development is apparent after 2.5 h in the assay used. The method has been successfully used in this laboratory for samples of sorghum grain with relatively high tannin contents. It is much less reliable in our estimation in cases where the tannin content is low, and this is due to the side reactions noted and to other, as yet, undetermined colour-producing reactions that occur in the procedure.

β-Glucosidase Method

We have finally sought to develop an entirely new procedure for tannin assay in sorghum grain that utilizes the major property of these polyphenols, which may be classified as tannins, that distinguishes them from all other phenols — namely their ability to precipitate and complex with proteins. Various protein solutions have from time to time been proposed as test substances for the purpose of estimating vegetable tannins. Thus both gelatin and casein have been used, and Bate-Smith has utilized the proteins of hemolyzed blood to determine the relative astringency of some vegetable tannins. In this work the association of the vegetable tannins in sorghum grain with the enzyme β-glucosidase (EC 3.2 1.21) has been examined in a quantitative manner to discover if use can be made of this property to estimate the vegetable tannin content of sorghum grain.

β-glucosidase enzyme was extracted from sweet almonds and used as a freeze-dried powder (~ 4 units of enzyme activity/mg of protein ex-

Table 1. Analysis of tannin content of sorghum seeds.

Sorghum cultivar	Vanillin assay[1]	Ultra-violet method[1]	Cyanidin method[1]
BR 44	0.76, 0.70, 0.82	0.85, 0.80	0.80, 0.73, 0.71
BR 54	1.61, 1.49, 1.64	1.14, 1.24, 1.29	1.68, 1.62, 1.73
BR 64[2]	2.66, 2.48, 2.68	2.12, 2.20, 2.08	2.09, 2.15, 2.01
NK 300[2]	0.92, 0.88, 0.81	0.70, 0.75, 0.82	0.90, 0.94, 0.79
NK 300[3]	0.11, 0.15	—	0.02, 0.03
IS 2319[4]	0.14, 0.12	—	0.07, 0.06
IS 3648	2.95, 2.82, 2.69	2.38, 2.20, 2.41	2.80, 2.64, 2.73
IS 3924	0.09	—	0.04
IS 4225[2]	0.66, 0.59, 0.69	0.55, 0.50, 0.58	0.85, 0.74, 0.76
IS 9115	0.53, 0.58, 0.59	0.54, 0.49, 0.46	0.71, 0.67, 0.61
IS 9119[5]	0.14	—	0.06
IS 11167[5]	0.20, 0.15, 0.24	—	0.22, 0.20
IS 15991[2]	0.31, 0.28, 0.21	0.10, 0.12	0.16, 0.17
954114[3]	0.04	—	0.05
954206[4]	0.04	—	0.02
RS 671	0.02, 0.08	0.06	0.06

[1]Standard was the polymeric procyanidin isolated from Sorghum NK 300 supplied by Purdue University, Lafayette, Indiana. Figures given are percentage of dry weight of the whole sorghum seed.
[2]Coloured pericarp with coloured testa.
[3]White pericarp with white or no testa.
[4]White pericarp with coloured testa.
[5]Coloured pericarp with white or no testa.

tract). Sorghum grain was extracted into methanol as previously described, the methanol removed at 30 °C, and the residual polyphenols dissolved in 0.2 *M* acetate buffer, pH 5.0. Aliquots of the phenol solution are then taken and added to a solution of the enzyme (~ 2 mg per ml) in the same buffer. The precipitated protein is removed from the solution by brief centrifugation and the residual enzyme activity in the aqueous supernatant then determined by abstracting aliquots and adding these to a solution of p-nitrophenyl β-D-glucoside in acetate buffer (0.2 *M*, pH 5.0). The rate of release of the p-nitrophenol from the substrate was determined by adding aliquots at given time intervals to tris-HCl buffer (pH 8.5) and the extinction at 420 nm recorded. A plot of the p-nitrophenol formed against time gave a progress curve from which the initial reaction rate, and hence the enzyme activity remaining in solution, was determined. In this way the extent of precipitation of the β-glucosidase by the sorghum phenols was evaluated. This was compared to a standard precipitation curve of β-glucosidase prepared using differing concentrations of the procyanidin polymer derived from sorghum NK 300, and from this comparison the percentage of tannin in the unknown sorghum sample determined.

The method is a time-consuming one and, although it is only outlined above, it is very sensitive but requires considerable attention to detail. We have not been able to achieve the type of consistency that is shown in the other three analytical methods described above over the period in which we have been using the procedure. The inconsistencies are due, we presume, to the numerous stages that have to be carried out and these appear to introduce random errors. However, in so far as the procedure appears to measure only those phenols that precipitate proteins (in this case β-glucosidase), it may be said to be the only procedure that actually measures the concentration of vegetable tannins in the sorghum grain in a specific manner. It may therefore be of interest to note that this procedure gives values that are similar to those shown in the accompanying tables but which are generally lower by some 10–15%.

Extraction of Polyphenols in Sorghum

We have utilized throughout this work one method for the extraction of polyphenolic material from the sorghum grain — namely solubilization in methanol. Some questions may be raised as to whether this is the best method and whether it removes *all* the phenolic material from the grain. Regarding the first question, the answer is that we have tried a variety of solvents (ethanol, propanol, water, acetone, dimethylformamide) and found that methanol has the most useful pro-

Table 2. Analysis of tannin content of sorghum seeds.

Sorghum cultivar	Vanillin assay		Ultra-violet method		Cyanidin method	
	Catechin[1]	Polymer[1]	Catechin[1]	Polymer[1]	B-2[1]	Polymer[1]
IS 3648	11.00	2.95	2.97	2.38	5.8	2.8
IS 4225	2.5	0.66	0.70	0.55	1.6	0.85
IS 9115	1.64	0.53	0.68	0.54	1.33	0.71
BR 44	2.87	0.76	1.2	0.85	1.5	0.8
BR 54	6.0	1.61	1.42	1.14	3.14	1.68

[1]Standards in analytical procedure.

perties so far as subsequent operations are concerned. As to the second question, the answer is that methanol does not remove all the phenolic material, some appears to remain behind, and may well be quite insoluble or attached to carbohydrate or other polymers in the grain.

Factors Affecting the Polyphenols of Sorghum and Their Development and Location in the Sorghum Kernel

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In sorghum grain, polyphenols are located primarily in the pericarp and the testa. Genes regulating pericarp colour and presence of the testa exist. Bird-resistant sorghums have a pigmented testa containing condensed tannins that remain in the kernel at maturity. In nonbird-resistant sorghums, the testa is resorbed during maturation. The endosperm of some white, colourless sorghums contains colour precursors that turn greenish-black when exposed to alkali. Variation in colour, changes in structure during maturation, polyphenol content, and nutritive value of sorghum genotypes are reviewed.

The major intent of this paper is to review the kernel structure of sorghum and discuss the factors that affect the polyphenols present in the grain. No attempt is made to cover all of the literature on the polyphenols of sorghum as other papers cover other specific areas. Certain ideas recorded here are based on observations gained over the years and are presented to stimulate discussion and further elucidation of their validity.

Structure of Sorghum Kernel

Fig. 1 is a diagram of the major anatomical parts of a mature sorghum caryopsis. The major parts of the kernel are the pericarp, the endosperm, and the germ.

The pericarp of sorghum originates from the ovary wall, which becomes dry and adheres strongly to the mature ovule. In certain sorghums a pigmented layer is present beneath the pericarp. Sorghum pericarp is usually considered to have four major parts: the epicarp, the mesocarp, the cross-cell layer, and the tube-cell layer. Sometimes the epicarp is divided into additional parts, i.e. epidermis and hypodermis. Numerous mistakes have been made in the literature regarding these terms. Until 1974 the US grain standards for sorghum erroneously used the term seed coat to refer to the pericarp of sorghum. The term seed coat should be used to refer to the layer that is the mature ovule wall in sorghum. Several terms including testa, subcoat, undercoat, inner integument, outer integument, nucellar layer, and others appear in the literature to refer to the seed coat. Current US standards for sorghum use the term subcoat, which is a highly descriptive, useful term. The use of "subcoat" or "testa" is preferred

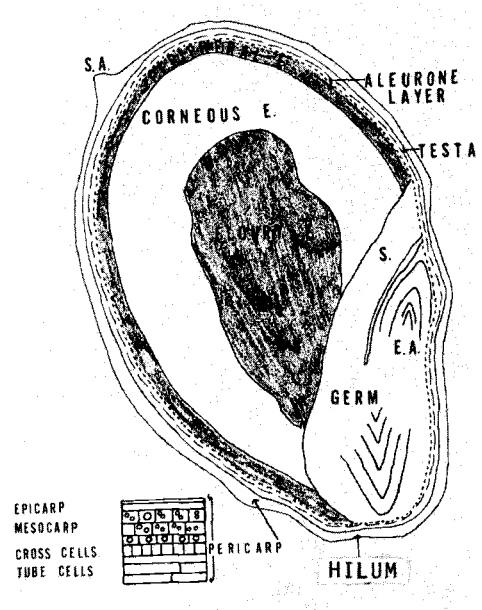

Fig. 1. Diagram of a sorghum caryopsis.

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Table 1. The genotype, phenotype, and market class of some common sorghum lines.

Variety	Testa	Spreader	Colour	Meso-carp	Endo-sperm	Plant colour	Testa	Pericarp	Endo-sperm	Plant colour	Appearance of kernel	Market class
BTx3197	$b_1b_1B_2B_2$	SS	RRyyii	zz	yeye	PPQQ	no	white, thick	nonyellow	purple	chalky white	white[2]
BTx378	$b_1b_1B_2B_2$	SS	RRYYII	zz	yeye	PPQQ	no	red, thick	nonyellow	purple	red	yellow
BTx399	$b_1b_1B_2B_2$	SS	RRYYii	zz	yeye	PPQQ	no	red, thick	nonyellow	purple	red	yellow
BTx398	$b_1b_1B_2B_2$	SS	RRYYii	zz	yeye	PPQQ	no	red, thick	nonyellow	purple	red	yellow
Pink Kafir	$b_1b_1B_2B_2$	SS	RRYYii	ZZ	yeye	PPQQ	no	red, thin	nonyellow	purple	light pinkish-red	yellow
Red Kafir	$b_1b_1B_2B_2$	SS	RRYYII	ZZ	yeye	PPQQ	no	red, thin w/inten.	nonyellow	purple	bright red	yellow
RTx7000	$b_1b_1B_2B_2$	SS	RRYYii	zz	yeye	PPQQ	no	red, thick	nonyellow	purple	red	yellow
RTx7078	$b_1b_1B_2B_2$	SS	RRYYii	zz	yeye	PPQQ	no	red, thick	nonyellow	purple	red	yellow
RTx414	$b_1b_1B_2B_2$	SS	RRYYii	zz	yeye	PPQQ	no	red, thick	nonyellow	purple	red	yellow
RTx09	$b_1b_1B_2B_2$	SS	RRyyii	zz	yeye	PPQQ	no	white, thick	nonyellow	purple	chalky white	white[2]
Double Dwarf Feterita	$B_1B_1B_2B_2$	ss	RRyyii	zz	yeye	PPQQ	yes	white, thick	nonyellow	purple	chalky white splotches	brown
RTx428	$b_1b_1B_2B_2$	SS	rryyII	ZZ	yeye	P-q^rq^r	no	white, thin w/inten.	nonyellow	red	white	white[2]
RTx430	$b_1b_2B_2B_2$	SS	rryyII	ZZ	YeYe	PPQQ	no	white, thin w/inten.	yellow	purple	yellow	yellow
RTx2536	$b_1b_1B_2B_2$	SS	rryyii	ZZ	YeYe	PPQQ	no	white, thin w/inten.	yellow	purple	yellow	yellow
RTx2566	$B_1B_1B_2B_2$	ss	RRYYII	ZZ	yeye	PPQQ	yes	white, thin	nonyellow	purple	reddish-brown	brown
RTx2567	$b_1b_1B_2B_2$	SS	rryyii	ZZ	YeYe	PPQQ	no	white, thin	yellow	purple	yellow	yellow
77CS2 (SC0170-6)	$b_1b_1B_2B_2$	SS	RRyyII	ZZ	yeye	P-q^rq^r	no	white, thin w/inten.	nonyellow	red	white, pearly	white[2]
Hegari (PI34911)	$B_1B_1B_2B_2$	ss	RRyyii	zz	yeye	PPQQ	yes	white, thick	nonyellow	purple	chalky white splotches	brown
Dobbs	$B_1B_1B_2B_2$	SS	RRyyii	zz	yeye	PPQQ	yes	white, thick	nonyellow	purple	brown	brown
Shallu	$B_1B_1b_2b_2$	SS	RRyyii	ZZ	yeye	ppqq	no	white, thin	nonyellow	tan	white, pearly	white[2]
SC0748-5	$b_1b_1B_2B_2$	SS	rrYYII	ZZ	yeye	PPQQ	no	yellow, thin w/inten.	nonyellow	purple	brownish-yellow	yellow
BTx615	$b_1b_1B_2B_2$	SS	RRyyii	zz	yeye	ppqq	no	white, thick	nonyellow[1] (waxy)	tan	chalky white	white[2]
NSA740	$b_1b_1B_2B_2$	SS	RRyyii	zz	yeye	PPQQ	no	white, thick	nonyellow (floury)	purple	chalky white	white[2]
F-104 sel	$b_1b_1B_2B_2$	SS	RRyyii	zz	yeye	PPQQ	no	white, thick	nonyellow (sugary)	purple	white	white[2]
SC0104 sel	$B_1B_1B_2B_2$	ss	RRYYII	zz	yeye	PPQQ	yes	red, thick, w/inten.	nonyellow	purple	dark reddish-brown	brown

[1]Contains waxy endosperm (wxwx) all others are nonwaxy (WxWx).
[2]The class could probably be yellow, currently; but, redefinition of the white class to permit stained kernels would let it be classified as white.

to avoid the confusion that exists among these terms. The presence of a testa is controlled by B_1 and B_2 genes. Most commercial sorghums grown in the United States do not have a testa.

Other structural features of the sorghum kernel affect polyphenol content. For instance, the hylar and stylar areas of the kernel are coloured even when the sorghum pericarp is colourless. The glumes contain polyphenols that sometimes affect the kernel especially under humid conditions or when rainfall occurs during kernel maturation.

The Appearance of Sorghum (Colour)

The visual appearance of sorghum is based upon several interacting factors that influence the colour of the kernel as viewed by the eye. The pericarp colour and thickness; the presence, colour, and thickness of the testa; and the colour of the endosperm can affect the visual appearance of sorghum colour. The descriptive terms used to refer to grain colour are confusing unless accompanied by a description of the genetics of the factors affecting grain colour.

Attempts to measure and classify the colour of sorghum have varied from visual rating systems to use of colour measurements with instruments (Khan et al. 1979). We have used the colour difference meter to measure colour of grains in terms of tristimulus readings. The use of tristimulus techniques is promising and may prove useful to predict the colour of products produced from the grain. Objective methods of classifying grain colour are needed to help avoid confusion about sorghum colour.

Genetics of Sorghum Colour

The genetics of grain colour are complex, but considerable information is available (Quinby and Martin 1954; Schertz and Stephens 1966; York 1976). A summary of the genetics of pericarp colour and thickness, plant colour, and endosperm colour for some sorghum varieties and lines is presented in Table 1. The genetics is based on experience with numerous crosses involving the sorghum lines and varieties listed in Table 1. It is presented to serve as a reference for future work. It is desirable that all other workers present the genetics of the material they are working with in their studies. Such information is useful in the interpretation of results.

Pericarp colour is determined by the following: B_1-B_2-S-R-Y-I-Z. The presence of the testa is controlled by the B_1-B_2- genes. If they are both dominant, the sorghum kernel has a pigmented testa. If one set of B_1B_2 genes is homozygous recessive (i.e. $b_1b_1B_2B_2$) then the pigmented testa is absent. The B_1B_2 genes affect pericarp colour because when they are dominant and a dominant spreader gene (S-) is present, brown pigments appear in the pericarp. The colour is "spread" from the testa into the epicarp of the pericarp. The shade of brown in the epicarp is conditioned by the R-Y-I- genes. Thus, it is important to know the genetics of the pericarp.

The R-Y- genes determine whether the pericarp is red, white, or lemon yellow. The pericarp is red when the genes are R-Y-. It is white when the genes are RRyy or rryy. It is lemon yellow when the genes are rrY-. The lemon yellow colour is bleached by the sun and usually turns a dull brownish-yellow colour by the time the grain has dried to harvest moisture. Often the lemon yellow colour is retained in the pericarp under the glumes. Yellow pericarp colour is not associated with yellow endosperm and should not be confused with it. The intensifier (I-) affects the intensity of the colour. For instance, a kernel with dominant R-Y- and I- genes will appear bright red when the genotype is R-Y-ii. The testa colour is purple for tptp and brown when Tp- (Casady 1975). Hegari and Feterita have the purple testa.

Mesocarp Thickness

The Z- genes control the thickness of the pericarp. A dominant Z- gene gives a sorghum with a thin pericarp. Homozygous recessive (zz) gives a thick pericarp. The thickness relates to the number of layers in the mesocarp and the presence of starch granules. The presence of a thick, starchy mesocarp causes the pericarp to mask the colour pigments present in the testa and endosperm. The thin pericarp (Z-) is translucent and permits colour of the testa and/or endosperm to affect the appearance of the kernel. The pearly sorghums have dominant Z genes and usually a high proportion of corneous endosperm. Chalky sorghum kernels are those with a thick, starchy mesocarp. The thickness of the pericarp (mesocarp) varies when recessive (zz) is present, which indicates there may be additional modifiers influencing pericarp thickness, i.e. the difference between Tx09 and BTx3197. This had led York (1977) to propose a new scheme for classifying the genes for pericarp colour and thickness. The material presented in Table 1 follows the old system (Schertz and Stephens 1966).

Endosperm Colour

Yellow endosperm sorghums that have high levels of carotenoid pigments exist. In true yellow endosperm sorghum varieties, the pericarp is thin and colourless (R-yy or rryy), the testa is absent, and the colour of the kernel appears yellow because of the carotenoid pigments in the endosperm. If the kernel has a thick mesocarp (zz) it will sometimes appear white because the yellow colour of the endosperm is masked by the thick mesocarp. Some sorghums are called bronze sorghums because they have a thin red pericarp, no testa, and a yellow endosperm. Thus the appearance of the kernel is bronze because the yellow endosperm shows through the thin red pericarp and modifies the colour. When a thick pericarp is present, the yellow endosperm colour is masked.

The aleurone of sorghum is usually colourless, although under certain circumstances of insect or disease attack, pigments have been observed in the aleurone layer (see Fig. 2C). A coloured aleurone exists in barley (*Hordeum vulgare*) and corn (*Zea mays*); but none has been reported for sorghum yet.

Glume and Plant Colour

Another important consideration is plant and glume colour which affects polyphenol content of the grain. Usually the plant colour is considered as purple or tan. The genes controlling plant colour are: P-Q-. Tan plant colour is recessive. Actually four colour variations are described: P-Q-, reddish-purple; P-qq, blackish-purple; P-q^rq^r, red; and ppqq, tan. Usually, for practical purposes, the plant colour is considered to be purple or tan. No attempt to distinguish among the other genotypes is made.

Glume colour is associated with plant colour. The best way to determine glume colour is to examine the colour of the inside of the glume after the kernel is removed. Glume colour is determined by the plant colour loci P and Q. Black- and red-glumed plants are P-, and mahogany- and sienna-glumed plants are recessive pp. The Q-locus conditions the shade of purple pigmentation present. Glumes that have intent red, black, or purple colour have a tendency to stain the sorghum kernel because the polyphenolic pigments leach into the pericarp. In addition, injury to the kernel during development, by insects or moulds, tends to produce coloured pigments that are related to plant colour.

Other Genes Affecting Pericarp Colour

Other genes have been reported that affect pericarp colour and many more probably exist in the world collection of sorghums. A gene (Pb-) that causes purple spots in the pericarp has been reported which may function in some commercial sorghums. For instance, new white pericarp sorghums released by the Texas Agricultural Experimental Station appear to have purple specks in the pericarp. Brown wash genes (Bw_1-Bw_2-) exist that have an effect on pericarp colour when B_1-B_2- genes are present with a homozygous recessive (ss) spreader. The brown colour washes through the pericarp, but it is different from the spreader genes. Another gene, sun red, (Rs_1-), causes the colour of the pericarp to become red when it is exposed to the sun during maturation. Then it disappears and the pericarp colour is determined by the R-Y-I- genes.

Market Class of Sorghums

In the United States Standards for sorghums, four classes of sorghum are recognized, yellow, white, brown, and mixed. The major class of sorghum in the US is the yellow class. The sorghum of commerce is usually US number 2 yellow sorghum. The market class of sorghum is determined by visual appearance of the grain; the presence of a pigmented testa means the grain goes into the brown class (Table 1).

The class, yellow sorghum, can contain grain of any colour pericarp as long as it does not contain a pigmented testa. Therefore yellow sorghum can contain grain with white, red, and lemon yellow colour pericarp. It cannot contain more than 10% of kernels with a pigmented testa. In practice, US yellow sorghum will contain almost no kernels with pigmented testa.

The white sorghum class contains kernels with 98% white pericarps without a testa. This class cannot contain more than 2% of nonwhite sorghum kernels. In addition, kernels with a white pericarp and spots are considered nonwhite kernels. In practice, the white class is not of importance in current marketing of sorghums, but it could become of more importance because new sorghum hybrids with high yield potential have a white pericarp without a testa. The white class will be most useful for maintenance of grain quality for food use where colour and flavour are prime concerns.

The brown sorghum class consists of kernels with a pigmented testa (B_1-B_2-) regardless of pericarp colour. The brown grain class has depressed

nutritional value compared to the yellow and white classes of sorghum. The bird-resistant high-tannin sorghums belong to the brown class. The most effective bird-resistant sorghums contain B_1-B_2-S-R-Y- genes. These genes appear to have the most serious effects on the nutritional value of sorghum. They consistently inhibit enzymes and contain high levels of condensed tannins. Other brown sorghums with a testa (B_1-B_2-ss) have variable effects on nutritional value. At this point, the safest, most conservative method is to continue placing all sorghums with a testa in the brown class. The overall quality of brown grain for feed and food is definitely lower than the yellow and white grains.

The yellow sorghum of US origin contains little if any condensed tannins and has nutritional value at least 95% equivalent of corn and greater for some species. Certain countries produce bird-resistant, mould-resistant, high-tannin sorghums. Problems in marketing arise when sorghums with high levels of tannins are sold as yellow sorghum. The buyers are not impressed with the performance of the sorghums and they develop a low opinion of all sorghums. Current efforts to educate the grain trade about sorghum quality variation include use of methods to detect the presence of a testa or undercoat. Educational programs have helped eliminate the production of bird-resistant sorghum in Australia and are reducing its production in Argentina. These comments are presented here because of their relevance to the overall question of tannins in sorghum.

The move toward production of white sorghum in the USA can be seen as a positive step toward utilization of sorghum in foods. The best way of solving the tannin problem is to grow white or yellow sorghums and discourage growth of brown where they are not really needed for bird resistance. Nonetheless, there are areas in the USA and elsewhere in the world where bird-resistant sorghums are required. Thus, there is an urgent need to understand the chemistry of sorghum-condensed tannins, to develop effective methods to eliminate or reduce their detrimental effects on nutritional value.

Methods of Tannin Analysis in Grain Marketing

Tannin analyses of sorghum are difficult even for research laboratories to perform. Substantial progress has been made toward better methods of polyphenol analysis of sorghum (Daiber 1975; Maxson and Rooney 1972; Price and Butler 1977; Price et al. 1978; Hagerman and Butler 1978). Application of these analytical methods to grain marketing situations is extremely difficult because of the need for simplicity, speed, reliability, and relation to nutritive value. No analysis comes close to meeting all these requirements, but the most effective for use in a grain marketing situation is the chlorox bleach method or pocket knife test to determine the number of kernels with a pigmented testa. The Federal Grain Inspection Service of the United States Department of Agriculture (USDA) detects brown sorghums by visual examination of the grain sample prior to and after scraping the kernel to look for the testa or subcoat. In certain instances, the chlorox bleach method is used as an unofficial aid to help determine the level of brown grain in sorghum samples, but it is not an official method.

For inexperienced personnel, the chlorox bleach method is a good method of determining whether a sample of sorghum contains grain with a pigmented testa. The percent of brown kernels in the sample can be used as a semi-quantitative estimate of the levels of condensed tannins. The chlorox bleach test was devised to remove the pericarp from cereals to facilitate estimates of germ damage (Weak et al. 1972). We have used the method for several years to detect brown sorghums in yellow sorghums. In 1975 the method was demonstrated to the grain industry in nine European countries and has been widely used since as an aid to determine the levels of brown grain (kernels with a testa) in market samples.

The chlorox bleach method is based on the removal of the pericarp which exposes the pigmented testa. In addition, the testa usually becomes an intense dark colour. Therefore the brown sorghum after bleaching is a dark colour, while nonbrown sorghums have a light colour. Usually it is relatively easy to distinguish between the brown and nonbrown sorghums, but weathering, leaching of pigments into the grain, insect and disease damage can cause nonbrown kernels to bleach darker than usual. Therefore reasonable care must be used in interpreting the results. Pericarp thickness and ease of removal varies among sorghum kernels within a variety and among varieties. In addition, testa thickness varies within kernels in a single variety. If the temperature, heating time, and methods of stirring are varied, erroneous results can be obtained because the testa can be completely or partially removed. Thus, it is important to set up the procedure and follow it carefully.

The following procedure had been found most useful in our laboratory for market samples. Potassium hydroxide 5.0 g is added to 15.0 g of

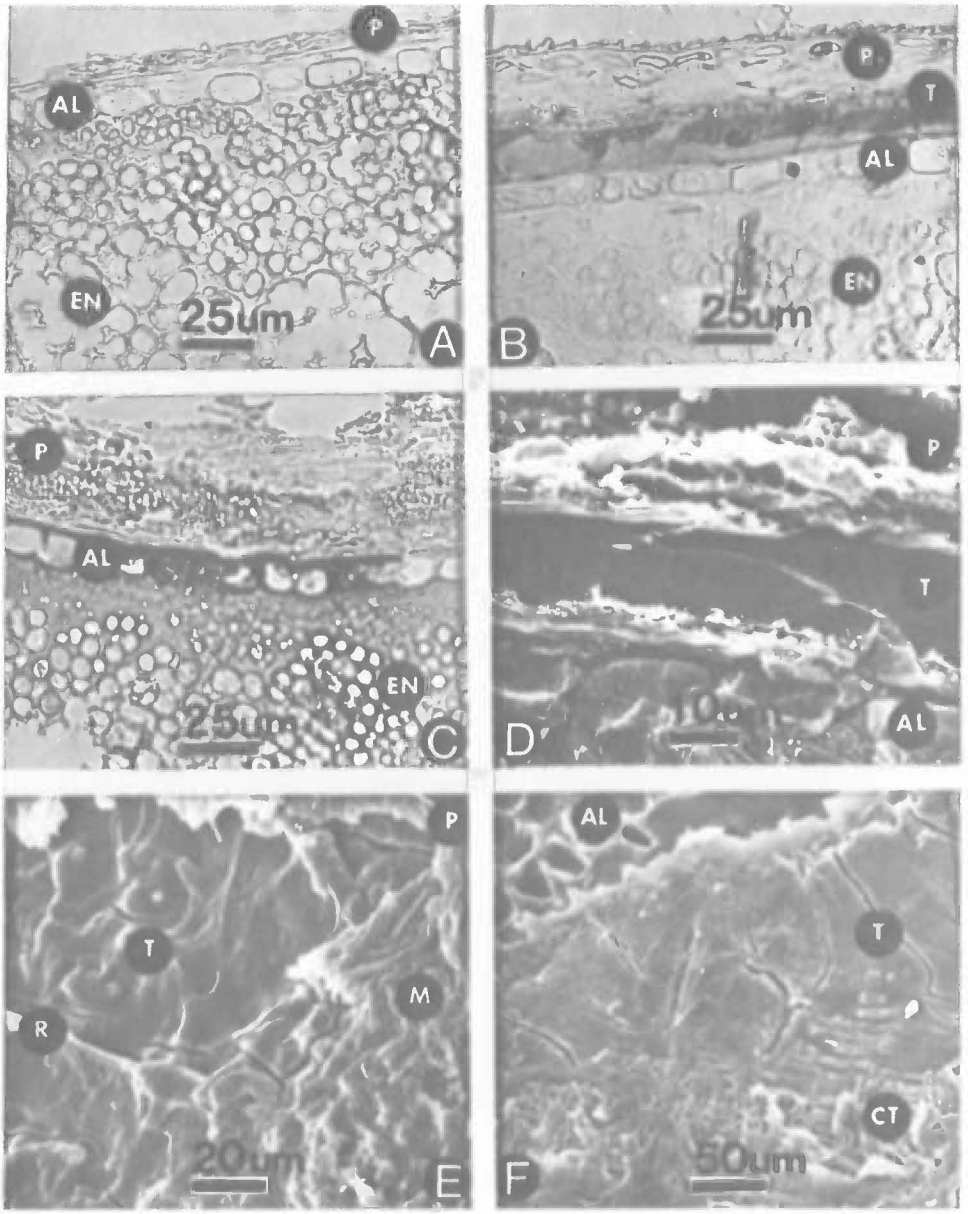

Fig. 2. Light and scanning photomicrographs of sorghum kernels. A) White pericarp without testa (400×). B) White pericarp with testa (400×). C) Red pericarp without testa, aleurone layer is stained (400×). D) SEM photograph of white pericarp with testa (1000×). E) SEM photograph of red pericarp with testa after partial removal of pericarp (500×). F) SEM photograph of red pericarp with testa after partial removal of pericarp (200×). AL-aleurone; CT-cross and tube cells; EN-endosperm; M-mesocarp; P-pericarp; R-ridge; T-testa.

sorghum in 70 ml of 5.25% aqueous sodium hypochlorite (NaOCl). The mixture is heated with stirring at 60 °C for 7.0 min. The sorghum is placed in a tea strainer and rinsed with tap water and dried. The procedure can be modified for use under different circumstances by using known reference samples of sorghums with and without the testa layer.

The US market class for sorghums with various gene combinations is presented in Table 1. The chlorox bleach test is most useful in the marketing of grains, but it can also be used to select for brown sorghums in breeding programs (Kofoid et al. 1978).

The Prussian blue test (Price and Butler 1977) has excellent potential for use in grain marketing, but it has the disadvantage of requiring uncommon reagents, the glassware is stained, and it works best on ground samples. We have not been able to achieve satisfactory results on whole kernels. None of the above disadvantages is major, but the chlorox bleach method is much simpler for use in the grain marketing system.

The chlorox bleach test can be used in marketing to determine the percentage of brown grain. A grain buyer can decide the price reduction for certain levels of brown kernels. In this way the chlorox bleach method can be used as an approximate index of the nutritive value of sorghum. Should a user of sorghum wish to determine the nutritive value of the common bird-resistant sorghums grown in the area from which the sorghum is received, a value can be arrived at by this method. Further work is needed to develop reliable methods of determining the content of condensed tannins in sorghum to be able to accurately predict its nutritional value.

The Structure of Sorghum Kernels with a Testa

We have examined numerous kinds of sorghums with light and scanning electron microscopy (SEM) to determine where the polyphenols were located. Fig. 2 is a comparison of the structure of kernels with and without the testa. The pericarp colour was $b_1b_1B_2B_2$ ssrryyiizz for the grain in Fig. 2A, while it was $B_1B_1B_2B_2$ ssrryyiizz for the grain in Fig. 2B. In other words both had a thick white pericarp. A part of the pericarp in Fig. 2A was lost during sectioning. Both appeared chalky white, but the one with a testa would be in the brown market class. We have not observed any sorghum kernels that contain an unpigmented testa layer. Although, in sections of many kernels without a pigmented testa, we have seen a remnant tissue which might be classified as nonpigmented material left from the integuments or nucellus.

The testa sometimes appears as distinct blocky cells consisting of two overlapping layers (Fig. 2B). The layers in Fig. 2B are of different colour intensity. One layer was reddish-brown while the other was a light yellowish-brown when viewed with bright field microscopy without staining. Usually the proportions of the two layers do not remain constant around the kernel. In other sections, the testa appeared as a single strip without structure, whereas in yet others blocky subunits were observed. Fig. 2D is a cross section viewed with SEM. No evidence of intracellular structure in the testa was observed even after attempts were made to extract the tannins with various solvents. We have not examined the immature testa with SEM. A top view of the mature testa is presented in Fig. 2E and F. This section was prepared by removing the pericarp after the kernel was softened with ethanol. In some kernels the testa appeared to have ridges which rose above the main plane of the testa (Fig. 2E). However, in others the ridges were not observed.

The thickness of the testa varies around the kernel (Blakely et al. 1979). The thickest areas (100–140 μm) occur just below the stylar region (Fig. 3) whereas the thinnest region (10–30 μm) is located on the opposite side of the hilum. The structure of the stylar and hilum is presented in Fig. 3. The hilum (hilar region) appeared to be a continuation of the testa, but the pigments are dark black. At the hilum, the testa and aleurone layers tend to be meshed together. The pigments are probably different from those of the testa. Mean and range of testa thickness for five varieties measured at each of eight specified locations on the kernel were presented by Blakely et al. (1979). The means ranged from 18 μm to 150 μm.

We have found a sorghum kernel that has a partial testa. The genetics are unknown, but the partial testa characteristics can be manipulated. Photos of kernels with a partial testa are presented in Fig. 4. Sorghum kernels with a partial testa have been reported (Quinby and Martin 1954), but they have been difficult to confirm. However, this partial testa is easily confirmed because the pericarp is thin. Fig. 4 C and D were sections from kernels with the partial testa. The testa disappears (Fig. 4 C and D). Other parts of the kernel such as the aleurone and pericarp are unchanged. The testa, in the kernels with a partial testa, appeared as small blocky units rather than a smooth stripe. When the testa was absent, the structure was similar to that of kernels without the testa.

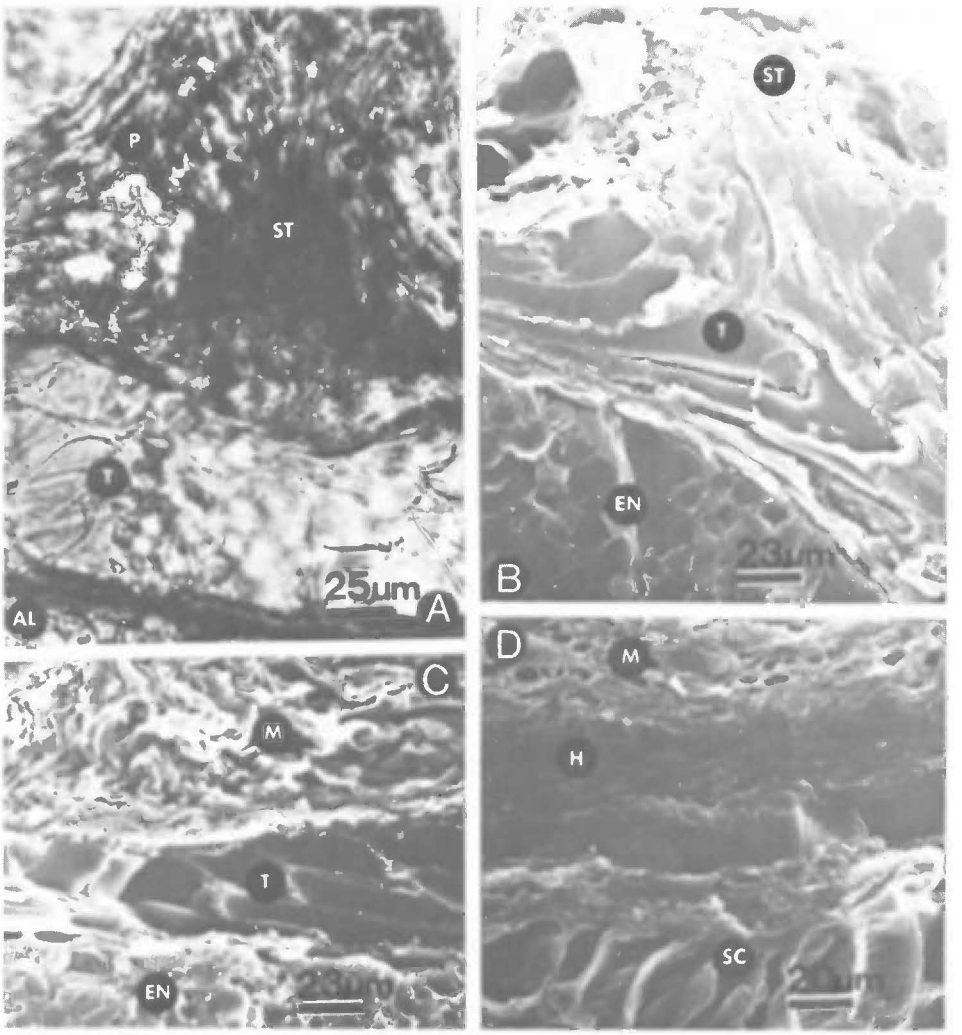

Fig. 3. Stylar and hilar areas of sorghum kernels. A) Stylar region of a kernel with red pericarp with testa (400×). B) SEM photograph of stylar region of white pericarp with testa (430×). C) SEM photograph of stylar region of red pericarp with testa (430×). D) SEM photograph of hilum region of brown (appearing) pericarp with testa (500×). AL-aleurone; EN-endosperm; H-hilum; M-mesocarp; P-pericarp; SC-scutellum; ST-style; T-testa.

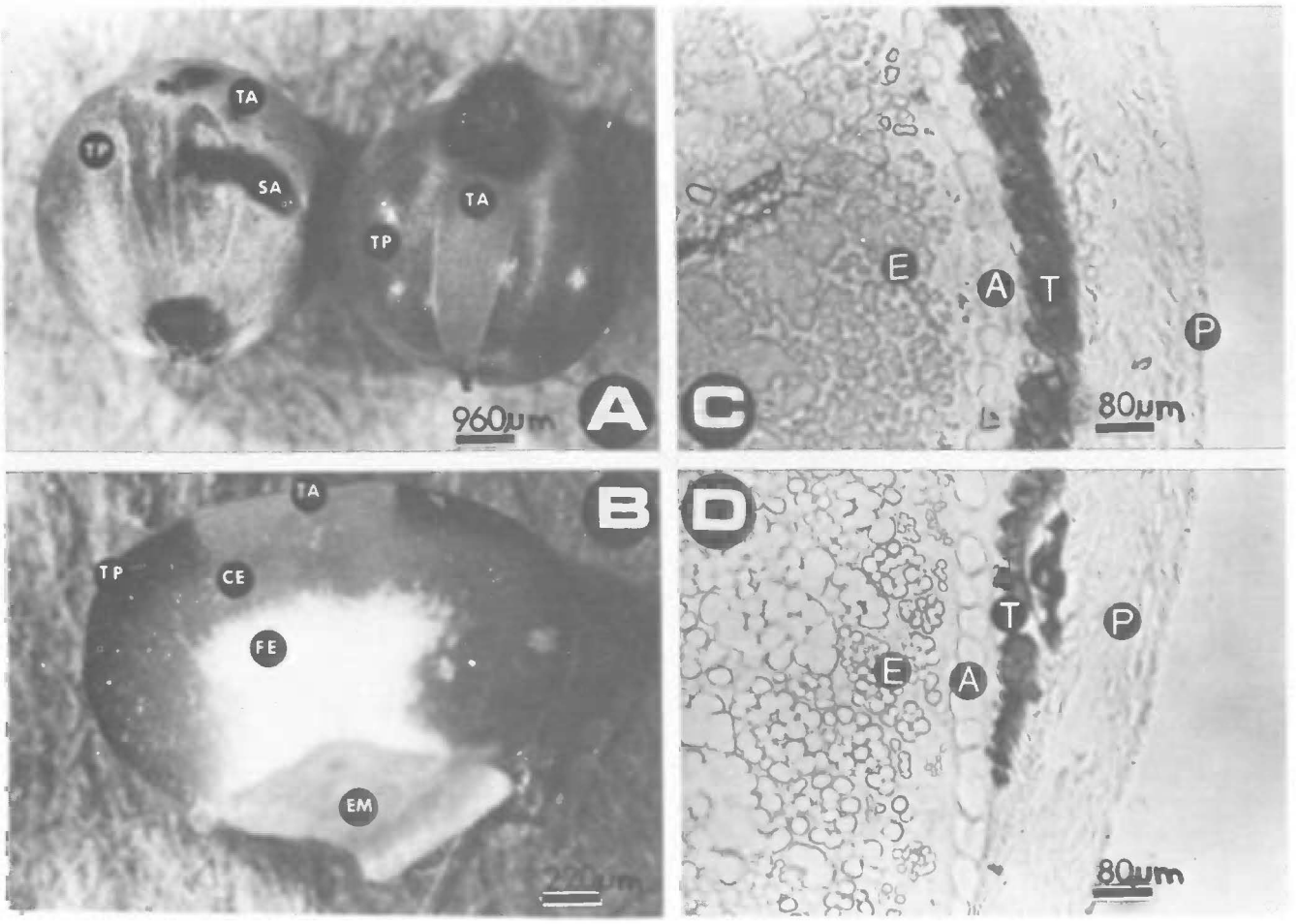

Fig. 4. Kernels with a partial testa. A) Whole kernels. B) Half kernels; the endosperm is unstained, the light is reflecting off the testa which gives the apparent darkness. C&D) Continuous sections of the testa ending in a partial testa line (500×). A-aleurone; CE-corneous endosperm; E-endosperm; EM-embryo; FE-floury endosperm; P-pericarp; SA-stained area; T-testa; TA-testa absent; TP-testa present.

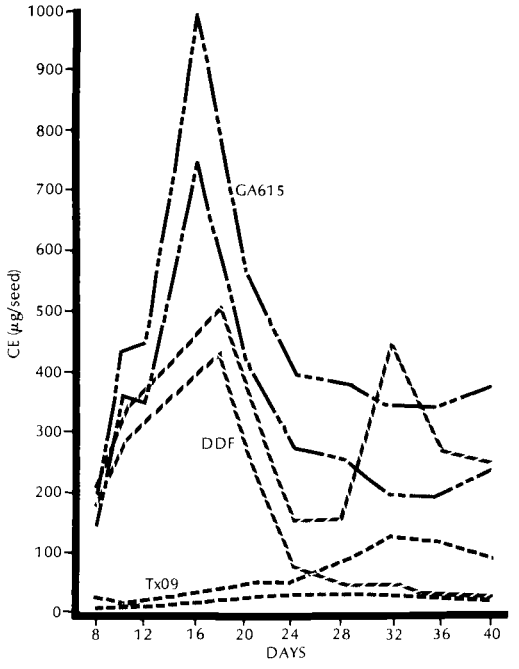

Fig. 5. The variation in catechin content of kernels from three sorghum varieties from anthesis to maturity. The catechin equivalents were determined with the vanillin method on 1% HCl methanol extracts of the grain. Catechin equivalents were calculated without subtracting a blank and after subtracting a blank. Hence, two curves exist for each variety.

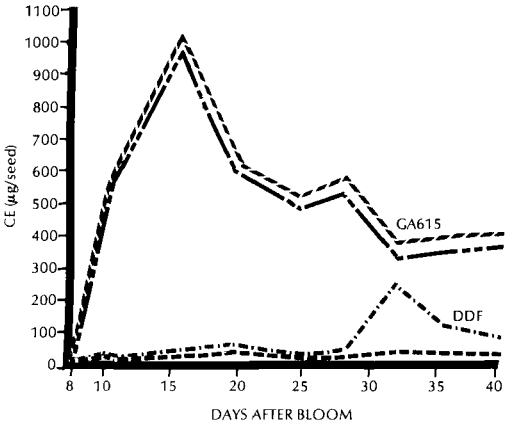

Fig. 6. The variation in catechin content of kernels from two sorghum varieties from anthesis to maturity. The catechin equivalents (CE) were determined with the vanillin method on methanol extracts of the grain. Catechin equivalents were calculated without subtracting a blank and after subtracting a blank. Hence, two curves exist for each variety. Tx09 did not have any measurable catechin equivalents. Hence, it is not on the graph.

Structure of Group II Sorghums

We have examined sorghum kernels that have been classified as group 11 (Price and Butler 1978) according to their polyphenols contents as measured with the vanillin (Price and Butler 1978) and modified vanillin methods (Maxson et al. 1972). The samples were sent to us by Dr John Axtel from Purdue. We have found what appear to be distinct differences in the structure of the testa among sorghums that were classified as group 11s. The testa of some group 11 sorghums appears lighter in colour and consists of two layers. One of the layers is colourless when viewed with bright field microscopy. The colourless layer varies in thickness and is between the testa and the aleurone cells. It is clearly a layer and one that we have not observed before. We feel it is not an artifact, but we have only looked at samples grown at one location. These sorghum samples form a dark colour when bleached with KOH and chlorox bleach (NaOCl). The colour of the

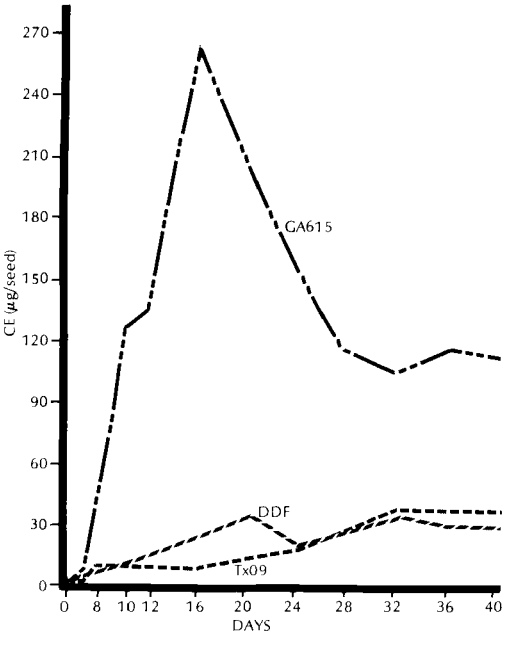

Fig. 7. The catechin equivalents of three kernels from three sorghum varieties from anthesis to maturity. The catechin equivalents (CE) were determined by the Prussian blue method (Price and Butler 1977).

bleached grain appears a little lighter for some samples than that of most sorghums with a testa. But the light colour may indicate incomplete removal of the thick, chalky pericarp over the testa and may not have anything to do with thickness of the testa. These observations are preliminary and further experiments must be made.

Grain Maturation Studies

A study to determine changes in the polyphenols and kernel structure during development and maturation of the kernel was made on three sorghum varieties. Florets were tagged and samples of individual florets were taken at anthesis (bloom) and at 1, 2, 4, 6, 8, 10, 12, 16, 20, 24, 32, 36, and 40 days subsequently for microscopy and chemical analysis. The varieties included: Tx09 ($b_1b_1B_2B_2$ ss rryyiizz PP QQ) a white, chalky nonyellow sorghum; Double Dwarf Feterita ($B_1B_1B_2B_2$ ss rryyiizz PP QQ); Redlan x Combine Shallu ($B_1b_1B_2b_2$ SS R R YyiiZz Pp Qq).

Enough grain was harvested at 8, 10, 16, 20, 24, 28, 32, 36, and 40 days for tannin analyses by the Prussian blue method (Price and Butler 1977); the vanillin-HCl method applied on methanol extracts (Price and Butler 1978); the modified vanillin-HCl method (Maxson et al. 1972); and an enzyme inhibition method (Blakely et al. 1979). Data with and without blanks subtracted were obtained for both vanillin methods and are presented in Fig. 5 and 6.

The micrograms of catechin equivalents (CE) per seed are presented in Fig. 5, 6, and 7 for the vanillin and Prussian blue methods. Fig. 8 presents the α-amylase inhibition data. The GA615 grain had the maximum level of catechin equivalents at 16 days for all methods of chemical analysis; but the maximum for enzyme inhibition units per seed was 28 days after bloom. Grain from Double Dwarf Feterita (DDF) — a kernel with thick white pericarp and testa — contained about half the catechin equivalents of GA615 at 16 days when measured by the 1% HCl-methanol extraction procedure. However, when the extractant was only methanol, the catechin equivalents were essentially zero at 16–20 days, whereas a significant increase in catechin equivalents occurred at 32 days. At this point, the CE for GA615 had decreased by about 50% of the value reached at 16 days. Tx09, white without a testa, had essentially no catechin equivalents by the vanillin methods. Some materials in Tx09 and DDF gave a slight response to the Prussian blue method (Fig. 7).

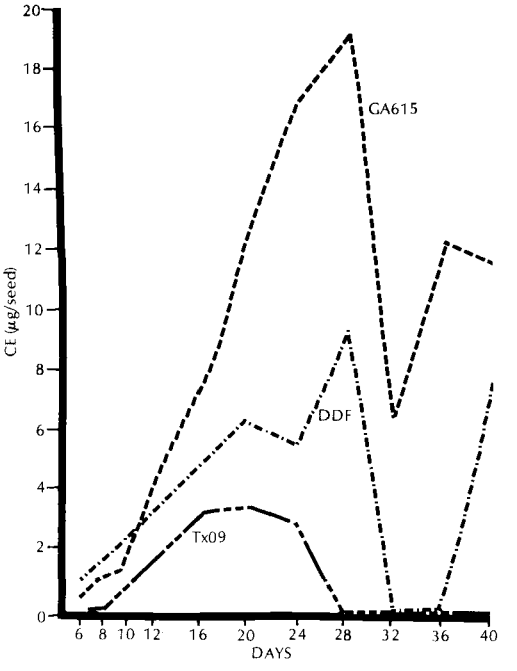

Fig. 8. Variation in α-amylase inhibition by water extracts of grain from three sorghums from anthesis to maturity.

The α-amylase inhibition test had a great deal of variability among laboratory replicates. It appeared that DDF had some increase in inhibitory power during maturation with a peak at 28 days followed by a sharp loss of inhibitory power upon maturation. The high variation of this method prevents conclusions about the changes in DDF. We do not consider the low levels of inhibition by Tx09 to be significant. The differences shown in Fig. 8 may not be repeatable. In previous experiments (Maxson et al. 1973) we found no inhibition of amylase by mature grain from Tx09 and DDF.

These data represent the results for only one season and should be considered as preliminary data. They are presented here to provide additional information on the effects of grain maturity on polyphenol compounds in the grain.

Biochemical Effects of Sorghum Polyphenols[1]

Larry G. Butler[2], Ann E. Hagerman[2], and Martin L. Price[2]

The ability of polyphenols present in ground sorghum grain to precipitate added soluble protein is strongly affected by pH, concentration, protein pI, and prior treatment of the grain (wetting, cooking, chemical "detoxification"). Fractions of purified sorghum polyphenols precipitate 15 times their weight of protein. Early in grain development, levels of methanol-extractable polyphenols are relatively high and diminish to various degrees during maturation. Freezing or brief heating of cut immature heads causes a rapid decrease in the amount of extractable polyphenols. Latest modifications of our polyphenol assays and techniques for obtaining protein-free polyphenol preparations will be described.

The diverse biological effects, both positive (agronomically) and negative (nutritionally), of condensed tannins (proanthocyanidins) in sorghum and other seed crops may have a common biochemical basis. The only well-established biochemical effect of these materials is that of protein binding and precipitation, which accounts for the astringent and enzyme-inhibitory properties of tannins. In order to investigate the biochemical effects of tannins, we have developed or adapted techniques for purifying sorghum tannin and for quantifying its ability to precipitate protein.

In our laboratory, when conventional procedures for purification of condensed tannins, including chromatography on Sephadex LH-20, are applied to sorghum grain, preparations containing considerable proportions of N (up to 3% of extract DWB) are obtained. Contaminating protein is presumably responsible for the nitrogen present. We have modified these preparative techniques to include a preliminary extraction with absolute ethanol resulting in only a small loss in tannin. The ethanol presumably removes alcohol-soluble proteins which would otherwise be extracted with the tannins by methanol. Both the ethanol and the subsequent methanol, which extracts the tannins, contain ascorbate as a reducing agent. After an ethyl-acetate extraction to remove most low MW phenolics and a batchwise adsorption cycle on LH-20, we extract an aqueous solution of the partially purified tannin with liquefied phenol, which removes most of the remaining protein. After column chromatography on LH-20, our product contains about 0.2–0.3% N (<2% protein). The yield is 20–30% of the phenolic material (Prussian blue assay, Price and Butler 1977) originally extracted, and the protein-precipitating power per phenolic unit is approximately double that in the original extract. The protein-precipitating power of the purified tannin is about 50 times greater than that of the same weight of grain from which it was obtained. This material is used as a standard in our tannin assays.

In assaying for protein precipitation by tannins, an aqueous solution of well-characterized protein, usually bovine serum albumin (BSA), is mixed with a solution of purified tannin or an extract of seed. The tannin precipitated is measured by a spectrophotometric assay after dissolving the precipitate in detergent (Hagerman and Butler 1978), and the protein precipitated is measured by using ^{125}I-labelled protein (Hagerman and Butler, in preparation). By measuring both components of the system, the conditions may be varied widely. The precipitation assay has been found to correspond well with the standard vanillin assay (Price et al. 1978). Standard curves are satisfactorily linear over a range of 0.1–2.0 mg tannin for both sorghum-condensed tannin and commercial tannic acid (a hydrolyzable tannin). When protein is in excess, both types of tannin precipitate about the same amount of BSA; when tannin is in excess, BSA binds somewhat less tannic acid than condensed tannin (Fig. 1). Titration of fixed amounts of sorghum-condensed tannins with increasing amounts of BSA demon-

[1]The research reported has been supported by USAID, Contract No. ta-C-1212.

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strated quantitative precipitation of BSA up to the saturation point, independent of the original amount of tannin. At saturation, purified sorghum tannin precipitates 6 mg BSA per mg tannin under these standard conditions. Assuming the proteins in sorghum seed are equivalent to BSA in their interaction with tannin, a high-tannin sorghum contains more than enough tannin to precipitate all the seed protein under the conditions of these experiments.

Protein precipitation by condensed tannins is strongly pH dependent, with maximal precipitation near the protein isoelectric point. Proteins vary considerably in both the pH range of maximum precipitation and in the breadth of that range. Pepsin (pI 1.0) precipitates from pH 2 to 6, BSA (pI 4.9) from pH 4 to 5, and lysozyme (pI 11.0) precipitates from pH 5 to 10. Precipitation is not entirely dependent on hydrogen bond formation with un-ionized phenolic hydroxyl groups. Methanol and ethanol both enhance the precipitation of BSA by condensed tannin, while dioxane and DMF have the opposite effect (Hagerman and Butler, in preparation). These observations are consistent with the occurrence of a nonpolar component in the tannin-protein interaction.

The tannin content of mature, harvested sorghum grain is used to specify high-, intermediate-, or low-tannin types. This is quite appropriate when tannin assays are used to predict the nutritional quality of the grain. But when used to evaluate the susceptibility to bird depredation, which is especially troublesome when the grain is immature, the measurements made on mature grain may be inadequate. The time of appearance of tannin may be of greater agronomic importance than the amount present at maturity. It was hoped that varieties could be found which contained high concentrations of tannin early in the grain development but which could be classified as low-tannin grains at maturity. A possible mechanism for a decrease in tannin content upon maturation is the conversion of the tannin to an insoluble form.

The amount of tannin extracted from several varieties of sorghum grain as a function of grain maturity is shown in Fig. 2. Tannin content rose sharply beginning about 10 days after half anthesis and reached a maximum within 20–30 days. The same data expressed on a per seed basis showed similar patterns but the age of maximum tannin content shifted to 25–40 days.

The grain for these assays had been kept frozen from the day of collection, then was blended in methanol 0.01 *M* ascorbic acid just prior to analysis of tannin by the vanillin assay. An alternative procedure of drying the grain immediately after sample collection was used by Davis and Hoseney (1979) in a similar study. It was predicted that such treatment might be accompanied by enzymatic and oxidative changes. Upon drying some of the frozen samples and assaying by the usual procedures for dry grain (Price et al. 1978), a decrease in the tannin content of the immature grains was observed. Further investigation into

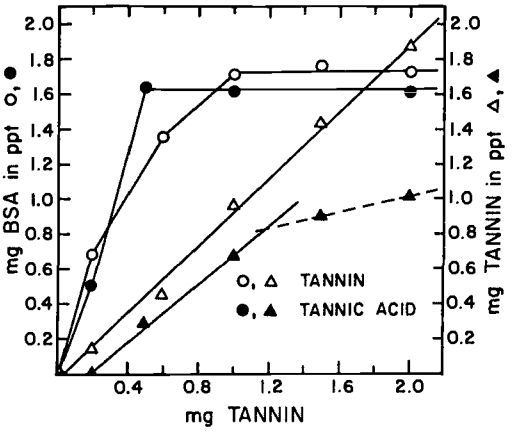

Fig. 1. Titration of 2 mg bovine serum albumin (BSA) with tannic acid or partially purified sorghum tannin. Aqueous solutions of tannin were added to the protein solution, pH 5, to make the final volume 4.0 ml. The tannin in the precipitate was determined by a spectrophotometric assay (Hagerman and Butler 1978) and the amount of protein measured by a radiochemical method (Hagerman and Butler, in preparation).

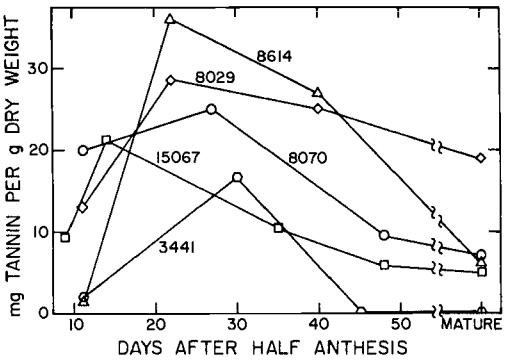

Fig. 2. Tannin content as a function of maturity for several varieties of sorghum. Methanol extracts of immature sorghum which had been stored frozen were assayed by the vanillin assay (Price et al. 1978). Tannin content is expressed on a dry weight basis. No tannin was detected at any time in several varieties (not shown).

Table 1. Effect of drying temperature on tannin content (%)[1] of immature grain.[2]

	Lyophilized	22 °C	40 °C	60 °C	105 °C
Savannah					
Untreated	—	4.86	3.89	4.14	0.68
Frozen	4.07	0.97	0.40	0.22	0.47
Boiled	—	0.72	0.25	0.97	0.90
IS-6881					
Untreated	—	4.82	4.14	3.67	0.11
Frozen	1.48	0.07	0.00	0.04	0.04
Boiled	—	0.11	0.72	0.00	0.11
IS-3441					
Untreated	—	2.84	1.94	1.30	0.00
Frozen	0.07	0.00	0.00	0.00	0.04
Boiled	—	0.04	0.04	0.04	0.00

[1]Mature Savannah later harvested from the same field had 2.05% tannin. Both IS-6881 and IS-3441 froze before reaching maturity.

[2]Heads of grain at 16-21 days after half anthesis were treated in one of three ways within an hour after cutting. Untreated: heads were subjected to no treatment other than drying at the temperature indicated. Frozen: heads were frozen and kept frozen for 5 days before drying as indicated. Boiled: heads placed in boiling water for 3 min before drying as indicated.

the nature of the decrease in assayable tannin upon drying frozen immature grain led to the surprising conclusion that drying fresh immature grain near room temperature did not result in a loss of tannin. Drying causes a decrease in tannin only if the immature grain has first been frozen or boiled. Fresh immature Savannah II and IS 6881, dried at room temperature, contained more tannin per gram of grain than was previously found in a mature grain (see Table 1).

The large decrease in assayable tannin on drying, that occurred only if preceded by freezing or boiling, implicates disruption of cellular compartments in the process. On disruption, tannin could become accessible to protein or some other cellular component and form insoluble complexes, which on drying would be even more strongly bound. Because freezing and boiling had similar effects, enzyme-catalyzed polymerization reactions after disruption are unlikely. A rat-feeding trial on immature high-tannin grain in which the assayable tannin was reduced by freezing, then drying, did not show a significant increase in rat weight gains or feed/gain ratio. But even the untreated grain, which contained 3.5% assayable tannin, gave large weight gains comparable to those seen with low-tannin sorghum. Because a similar diet of mature sorghum containing only 2% tannin produced 35% less weight gain than a low-tannin sorghum (Price et al. 1979), either the tannin in this variety (IS 6881) of sorghum is of no nutritional significance when the grain is immature, or some other component of the immature grain fully compensated for the antinutritional effect of the large amount of tannin present. The tannin extracted from this immature grain was even more effective in the protein precipitation assay than the tannin from mature grain of other varieties. This result suggests that protein precipitation may not be the only basis for the antinutritional effect of tannins.

The basis for the large decreases in assayable tannin observed in several sorghum varieties during maturation is of considerable interest. It is possible that these are the result of changes in the solubility of the tannins due to increased polymerization or association with some other cellular component (Price et al., submitted to J. Agric. Food Chem.). These effects are of considerable interest with respect to protection against bird depredation and to the antinutritional effects of tannins.

Treatments of Sorghum Grain That Reduce the Assayable Tannin Content and Their Effect on the Nutritional Value of the Grain[1]

Martin L. Price[2] and Larry G. Butler[2]

A variety of mild alkaline treatments of high-tannin sorghum grain lowered polyphenol content without extracting any components. Marked improvements in weight gain and feed efficiency were obtained by moistening the grain with dilute NH_4OH or 0.5 *M* K_2CO_3. Baking chapaties of high-tannin grain also caused a marked decrease in assayable polyphenol content. However, rats fed a cooked high-tannin grain showed poorer weight gains than did rats fed uncooked high-tannin grain. Rats fed cooked or uncooked low-tannin sorghum performed equally well—significantly better than those fed high-tannin grain.

Japanese sorghum grain importers have been more disturbed than usual this season with imports from Argentina because the tannin content is higher than normal (J. Kishida, personal communication). This was caused by a rainy season near harvest which resulted in substantial losses of all but the high-tannin types (Horacio Pacagnini, personal communication). Approximately two acres of sorghum that we planted in Puerto Rico this spring were wiped out entirely by birds, except for high-tannin types which were scarcely affected. These are two recent, dramatic reminders of the agronomic advantages of growing high-tannin sorghum in regions subject to bird damage or wet weather near harvest. On the other hand, the deleterious nutritional effects of tannin are well known (Price and Butler 1979).

It was found that treatment for several days with ammonium hydroxide, or for an hour with gaseous ammonia under pressure, reduced the assayable tannin in high-tannin sorghum grain by over 90%. Feeding trials with chickens showed that such reductions in tannin were accompanied by improvements in nutritional value, especially for the milder treatments. Table 1 presents 3-week-old chick and 4-week-old rat weight gain on diets composed of BR-54 (high-tannin) or RS-610 (low-tannin) which had been moistened with concentrated ammonium hydroxide at the rate of 10 ml for every gram of whole grain and allowed to stand in a closed container for one week. The grain was subsequently spread in a thin layer to allow ammonia to evaporate and then ground and mixed in the rations. Both weight gain and feed efficiency were markedly improved for the BR-54 by this treatment. It is unclear why the RS-610 was harmed by the treatment.

A different high-tannin grain (Savannah) and RS-610 were treated with dilute ammonium hydroxide prepared by mixing one part of concentrated NH_4OH with six parts of water (same volume/weight ratio as above) for one month at room temperature inside sealed plastic bags. After drying grain for one day at 50 °C, it was ground and mixed in rations. The body weight gains and feed efficiencies of 3-week-old chicks along with tannin analyses are shown in Table 2. Again substantial improvements in the nutritional quality of the high-tannin grain were observed, and this time no significant change was found in the quality of the low-tannin grain. However, the treatment time needed to substantially reduce the assayable tannin was of necessity much longer with dilute than with concentrated ammonia. All of the treatments described so far were on whole grain, which has advantages for drying, storing and transporting the grain.

Subsequent investigations demonstrated that the decrease in assayable tannin could be achieved by a variety of aqueous alkalies, and that grinding the grain decreased the time required for the treatment. Grain (18 kg) was ground, mixed with 3.6 litres of 0.5 *M* K_2CO_3 and kept at 52 °C for 42 hours in sealed plastic bags, then three days later dried as described above. The weight gains of chicks fed rations based on

[1]The research reported has been supported by USAID, Contract No. ta-C-1212.

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Table 1. Three-week-old chick and four-week-old rat feeding trials with sorghum grain (BR-54 and RS-610).[1]

Treatment[2]	% Decrease in assayable tannin	3-week-old chick		4-week-old rat	
		Weight gain[3] (g)	Feed/ gain[3]	Weight gain[3] (g)	Feed/ gain[3]
BR-54					
None	—	163*b*	2.7*b*	36*a*	7.1*a*
NH_4OH	72	247*a*	2.1*a*	63*b*	5.1*a*
RS-610					
None	—	258*a*	1.9*a*	58*a*	5.0*a*
NH_4OH	—	191*b*	2.1*a*	49*a*	5.3*a*

[1]Adapted from Price et al. 1978.
[2]Grain treated for 1 week with conc. NH_4OH.
[3]Values followed by different letters are significantly different ($p < 0.05$).

Table 2. Three-week-old chick feeding trial (Savannah and RS-610).[1]

Treatment[2]	% Tannin	3-week-old chick	
		Weight gain[3] (g)	Feed/ gain[3]
Savannah			
None	2.5	183*a*	2.32*a*
dil. NH_4OH	0.4	268*b*	1.96*b*
RS-610			
None	—	250*b*	1.87*bc*
dil. NH_4OH	—	268*b*	1.82*c*

[1]Adapted from Price et al. 1979.
[2]Grain treated for 30 days with dil. NH_4OH.
[3]Values followed by different letters are significantly different ($p < 0.05$).

Table 3. Three-week-old chick feeding trial with sorghum grain (BR-54 and RS-610).[1]

Treatment[2]	% Decrease in assayable tannin	3-week-old chick	
		Weight gain[3] (g)	Feed/ gain[3]
BR-54			
None	0	174*c*	2.54*a*
K_2CO_3	99	263*a*	2.10*b*
RS-610			
None	—	266*a*	1.84*d*
K_2CO_3	—	218*b*	1.97*c*

[1]Adapted from Price et al. 1979.
[2]Grain treated for 30 days with 0.5 *M* K_2CO_3.
[3]Values followed by different letters are significantly different ($p < 0.05$).

the treated high-tannin grain (BR-54) were substantially improved in comparison with the untreated (Table 3). The control treatment harmed the quality of the low-tannin grain somewhat, but it should be remembered that low-tannin grain would not be treated in practice but only as a control in scientific experiments.

The surprising lability of tannin to a variety of chemical treatments suggested the possibility that some or perhaps even most of the conditions prevailing during cooking sorghum for human consumption might similarly reduce or overcome the harmful nutritional effects of tannin. In many areas of the world where sorghum is used as food for the human population, there is a definite preference for light-coloured and hence low-tannin varieties of sorghum. But these preferences might be overcome in favour of high-tannin grain if the yields of the latter were dramatically higher and more reliable. For the present, no one feels free to recommend the high-tannin route for human consumption because of the likely nutritional consequences. If it could be demonstrated that certain cooking procedures effectively reduced the tannin content, the greatest impediment to their use would be the, perhaps considerable, barrier of human preference.

The original intention of this research was to use one of the common chemical assays to monitor the effect of tannin on cooking, using various recipes. (See Vogel and Graham 1979 for details of many food preparation methods currently in use.) It was found, however, that even moistening ground sorghum to prepare a batter resulted in an apparent loss of much of the tannin. We believe that this "loss" is caused by the formation of an insoluble complex between tannin and protein. Evidence for this view is presented in Fig. 1.

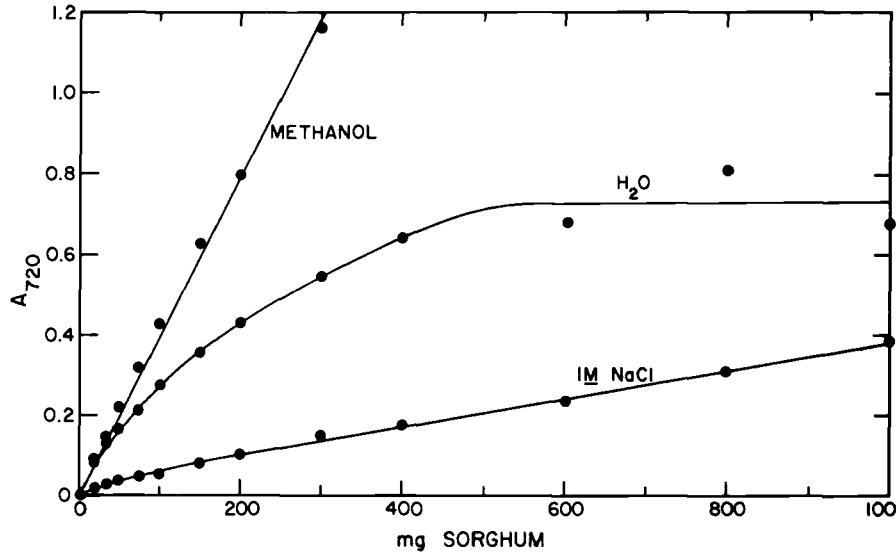

Fig. 1. Absorbance obtained with the Prussian blue assay (Price and Butler 1977) versus mg of sorghum grain per 10 ml of solvent for extraction into methanol, water, or 1.0 M NaCl (adapted from Price et al. 1979).

Capped test tubes containing 10 ml of either water or methanol and varying amounts of ground sorghum grain were rotated for 10 min to extract the tannin. When absorbance due to oxidizable components (tannin and probably other phenols) was plotted against the weight of sorghum in the 10 ml of solvent, a straight line was obtained for methanol. This is consistent with the fact that no insoluble tannin-protein complex formed in that solvent. At very low grain/solvent ratios, water contained nearly as much oxidizable material as did methanol, but this rapidly dropped below the quantities found in methanol as greater amounts of grain were added, and increasing concentrations of tannin and protein were presumably initially attained in the solution.

Price and Butler (1977) presented evidence that by using 1 *M* aqueous NaCl as solvent, the non-tannin oxidizable material could be selectively extracted from sorghum grain. If so, because none of this fraction of the oxidizable material should precipitate protein, it was predicted that a linear relation between absorbance and weight of sorghum would result when salt water was the extractant. This was found to be the case (Fig. 1).

Because it was not possible to monitor "detoxification" of tannin during cooking, it was decided to choose one cooking procedure, followed by feeding trials. Rats were fed diets for four weeks containing only sorghum, vitamins, minerals, and lysine-HCl. The sorghum in one diet had been ground, made into a batter, then baked into thin cakes by cooking 25 min per side in an electric skillet set at 400 °F, dried, and reground. A second diet was based on batter that had been dried at room temperature and reground. A third diet was an untreated control. Both Savannah and RS-610, high- and low-tannin grains, respectively, were tested. Rather than improving the nutritional quality, both treatments caused significant depression in weight gains of rats fed the high-tannin grain (Table 4). None of the treatments affected the low-tannin grain, which gave weight gains significantly higher than the untreated high-tannin grain.

A possible explanation for this decrease in the already low nutritional quality of the treated high-tannin sorghum, while the low-tannin sorghum was unaffected, could be that the tannin complexes with higher quality embryo protein, which is more soluble in water than is the lower quality endosperm protein. Thus the higher quality protein might contribute a disproportionate share to the tannin-protein complex which, if indigestible, would harm the quality of the grain. If whole grain were cooked *before* grinding, the tannins might be forced to complex with protein in the physical vicinity of the testa layer, which is likely to be of lower quality. Very little of the high quality embryo protein should be complexed, and the nutritional quality would be relatively good.

Savannah was boiled 10 min in water, in water containing 0.3% $NaHCO_3$, or in water for 30 min

Table 4. Effect of baking ground grain into chapaties or preparing chapati batter then drying, on measurable tannin content and on rat weight gain and feed efficiency.[1]

Treatment	% Tannin	4-week-old rat	
		Weight gain[2] (g)	Feed/ gain[2]
RS-610			
None	0.0	58.1*a*	4.85*a*
Batter	0.0	56.5*a*	5.12*a*
Chapaties	0.0	58.4*a*	5.17*a*
Savannah			
None	1.9	33.0*b*	8.89*b*
Batter	0.1	20.8*c*	12.7*b*
Chapaties	0.1	17.5*c*	11.8*b*

[1]Taken from Price et al. 1979.
[2]Means followed by different letters are significantly different ($p < 0.05$).

Table 5. Effect of cooking whole grain on measurable tannin content and on rat growth and feed efficiency.[1]

Treatment	% Tannin	4-week-old rat	
		Weight gain[2] (g)	Feed/ gain[2]
RS-610			
None	0.0	70.8*a*	4.83*a*
Savannah			
None	2.2	49.0*b*	5.69*b*
Boiled 10 min in H_2O	1.5	39.8*b*	6.83*b*
Soaked overnight Boiled 30 min in H_2O	0.9	36.4*b*	7.06*b*
Boiled 10 min in 0.3% $NaHCO_3$	0.9	40.1*b*	6.66*b*

[1]Taken from Price et al. 1979.
[2]Means followed by different letters are significantly different ($p < 0.05$).

after having soaked overnight in water, then dried and prepared into diets as described above. Only untreated RS-610 was fed this time, as the point of interest was whether or not Savannah could be improved. Four-week-old rats showed weight gains for Savannah that were significantly lower than for RS-610 (Table 5). All of the treated grains supported still lower weight gains, although differences between the Savannah diets were statistically significant only at $p < 0.1$. This was in spite of considerable decreases in assayable tannin after treatments.

It was then concluded that although various cooking procedures drastically reduce the level of assayable tannin in high-tannin sorghum grain, they do not overcome the deleterious nutritional effects of the tannin. On the contrary, there is evidence that cooking may be uniquely harmful to the nutritional quality of high-tannin grain. The identity of the protein which is complexed with tannin prior to feeding does not seem to be important. Feeding trials need to be conducted on several sorghum varieties before this apparent effect can be considered to be proven.

Sorghum Polyphenols and Bird Resistance

Roger W. Bullard[1] and Donald J. Elias[1]

Chemical, biochemical, and bird-repellent properties of polyphenols from a wide selection of sorghum varieties were evaluated. Several were evaluated in four stages of seed maturation. Also, the influence of molecular characteristics of 15 Sephadex LH-20 fractions were studied. Changes in sorghum polyphenol properties during maturation parallel those in other plant products. The molecular characteristics that influence chemical and biochemical properties of condensed tannins also appear to affect the degree of repellency to *Quelea quelea*. These studies may lead to development of sorghums with improved bird-repellent and nutritional characteristics.

Among North Americans and Europeans, sorghum is one of the least known of the world's major crops. Its primary use in the developed countries is almost exclusively as a feed grain and forage crop. But in some parts of the world (e.g. India, the Sahelian zone of Africa, the Near East, and Middle East) sorghum is a staple food crop and forms the basis of nourishment for much of the human population (Martin 1970). In 1977 sorghum was grown on 44 million hectares, of which 37 million hectares were in developing countries (FAO 1978). The reliance on sorghum as a staple food crop in many areas is due to its ability to withstand relatively harsh ecological and growing conditions (Doggett 1970). In some arid regions (e.g. the Sahel of Africa) it is the only cereal grain that can be grown with reasonable success.

Like all crops, sorghum is affected by a number of pests, and granivorous (grain-eating) birds are among the more serious. The red-billed weaver (*Quelea quelea*) of Africa is, perhaps, the most classic example. It has the distinction of being the most numerous and possibly the most destructive bird in the world, comparable only to locusts in the amount of damage it causes. The *Quelea* population base in Africa is estimated to be between 10^9 and 10^{11} (Crook and Ward 1968). Other species in other parts of the world likewise cause significant losses in sorghum plantings. Blackbirds, starlings, and sparrows are the major problem birds in North America. In southern Asia, sparrows, parakeets, crows, mynas, and several others cause havoc to sorghum crops (Rachie 1970). Eared doves, blackbirds, sparrows, parakeets, and parrots are the major problem species to sorghum growers in several Latin American countries. Hence, a great deal of interest is turned towards developing some means of alleviating the bird damage problem.

In areas such as Africa and many of the developing countries, the risks of such losses to birds are high. Agricultural practices are more labour intensive than capital intensive, hence, damage control technologies should be suited to these situations. Because chemical repellents or avicides are expensive, often difficult to obtain in developing countries, and require special equipment for application, it would seem more practical to place emphasis on building an ability to resist bird depredation into the genetic composition of the plants (Cummings 1976). Much effort has been directed to this end in sorghum. In fact, bird damage problems are often so severe that development of bird-resistant (BR) inbreds and hybrids became an integral part of many sorghum research programs during the 1960s and early 1970s (McMillian et al. 1972).

Bird resistance in sorghum has been attributed to several characteristics including the presence of stiff lemma awns (Boyd et al. 1965), lax panicles (Voight 1966; Perumal and Subramaniam 1973), and high tannin content (Fuller et al. 1966; Niehaus 1966; Tipton et al. 1970; Niehaus and Schmidt 1970). High tannin content is the characteristic most often associated with bird resistance and many data have been presented to support this relationship (Harris 1969; Tipton et al. 1970; McMillian et al. 1972). Several sorghum lines with high tannin content have been developed and promoted on the basis of their ability to resist depredation by birds. In fact, many of these bird-

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resistant sorghums are used extensively in those areas of the world where the risk of such losses is high.

The benefits to be gained, however, do not come without a price. Other researchers have found that the polyphenolics (tannins) which produce astringency, and thereby repellency, also reduce the palatability, digestibility, and nutritional quality of many foods containing them (Chang and Fuller 1964; Harris 1969; McGinty 1969; Glick and Joslyn 1970; Schaffert 1972; Cummings and Axtel 1973). One author (Morton 1970 and 1978) has proposed that there is a link between a high incidence of esophageal cancer in certain areas and the usage of tannin-containing plants within these areas. The result is that high-tannin sorghums have a lower value on the export market (Anonymous 1978), and farmers who must produce them because of high bird-depredation risk, are at an economic disadvantage.

There is an obvious need for further research and development in this area. Historically, most bird-resistant sorghum research has been restricted to field evaluations of damage. In view of the above, it seems reasonable to suggest that this approach is inadequate and that the factors affecting both bird resistance and nutritional quality in sorghum must be looked at from a different viewpoint. The purpose of the present paper is to review knowledge of those factors, chemical and biochemical, which influence the bird-resistant and nutritional characteristics of sorghum, and to examine how they may be utilized in the development of varieties that will adequately fulfill the needs of sorghum farmers around the world.

Classification of Sorghum Tannins

A review of the literature covering the past 20 years of sorghum research and development reveals a fascinating progression of understanding of the properties of polyphenolic tannins and their role in chemical bird resistance. For example, before 1970 most laboratories used tannic acid and other hydrolyzable tannins as standards for comparison in chemical and nutritional studies of sorghum tannins. Today, most agree that hydrolyzable tannins, if present, are found only in trace quantities, and that condensed tannins are responsible for the bulk of protein-binding activity (Bate-Smith and Rasper 1969; Strumeyer and Malin 1975). Although the term bitter has often been used to describe a taste property of sorghum polyphenols, most workers attribute bird resistance to the tactile astringent response. Astringency is that mouth-puckering sensation that results from coagulation of the proteins of the saliva and mucous epithelium when in contact with tannins (Joslyn and Goldstein 1964).

Condensed tannins are condensation products of flavan-3,4-diols with some incorporation of flavan-3-ols, primarily catechin or epicatechin. The biochemical activity of the polyphenol molecule depends on its ability to bind with various proteins. Such interactions are manifested in enzyme, fungi, and viral inhibition; in the tanning of hides; in lowered nutritional quality; and in the astringent taste response.

Flavan-3,4-diols are known to produce coloured anthocyanidins on heating with dilute acid (Ribereau-Gayon 1972). Several of these anthocyanidin-generating compounds have been identified in various sorghum seeds. However, there has been a general tendency not to distinguish between those that condense to form tannins and those that serve only as pigments.

The use of chemical analyses to distinguish between these proanthocyanidin tannins and nontannins can be misleading. Even the use of the favoured vanillin assays will detect anthocyanins (Sarker and Howarth 1976) or any proanthocyanidin that contains an unsubstituted phloroglucinol or resorcinol structure (Ribereau-Gayon 1972) whether or not they readily condense into tannins.

Profisetinidin was found in the pericarp of Martin sorghum (Blessin et al. 1963) and propelaragonidin in the pericarp of a "commercial sorghum" (Yasumatsu et al. 1965) but neither of these proanthocyanidins was tested for protein-binding properties. Bate-Smith and Rasper (1969) identified the principal "tannin" of a Kafir variety as luteoforol or proluteolinidin, which is a flavan-4-ol. They and Haslam (1977) reported the appearance of a red or pink-red pigment, stated to be mostly luteolinidin, as the green seeds begin to ripen. The authors of this paper and others (Strumeyer and Malin 1975) have found a similarly coloured pigment in the nontannin fraction from Sephadex LH-20 gel permeation chromatographic separation of polyphenolic extracts from BR (bird-resistant) sorghums. Unfortunately, all of Bate-Smith and Rasper's determinations of astringency were for the synthetic product of the borohydride reduction of eriodictyol and not an isolated natural tannin. Folin-Denis analyses which detect any phenols present (Goldstein and Swain 1963) were used for all of their tannin determinations.

The fact that Bate-Smith and Rasper (1969) reported luteoforol values for most sorghum samples, whereas only a few off-coloured

(bronze) seeds from their Kafir sample contained a testa, leads us to suspect that they were dealing with a nontannin pigment. Most investigators agree that a testa must be present for a variety to have significant quantities of tannin (Harris 1969; Maxson et al. 1972; Price and Butler 1977). Conversely, pericarp colour or pigmentation is not necessarily related to tannin content or biochemical activity (Thayer et al. 1957; Blessin et al. 1963; Stephenson et al. 1968; Harris 1969; Damron et al. 1968; Maxson et al. 1972 and 1973; Mabbayad and Tipton 1975, Nelson et al 1975). Red pericarp colour is controlled by two independent genetic factors Y and R (Wanjari and York 1972; Kambal and Bate-Smith 1976) while B_1B_2S controls the brown pericarp, the presence or absence of a pigmented testa, and tannin content (Maxson et al. 1972; Wanjari and York 1972).

All of this leads to the point that procyanidin chemistry is most likely involved in the bulk of the reported chemical, biochemical, and nutritional investigations of BR sorghums. This becomes apparent in reports of chemical investigations of testa-containing, high-tannin sorghum varieties. Strumeyer and Malin (1975) isolated the tannin fraction from Leoti and Georgia 615 varieties and found it to be exclusively procyanidin in nature. Haslam (1977) also found the tannins of NK 300 (Haslam, personal communication) to be procyanidin in nature.

Shape and Conformational Influences

Molecular shape is an important factor in determining the properties of procyanidin polymers (Quesnel 1968). Haslam and co-workers have made important contributions to the understanding of molecular configuration in procyanidin tannins. They categorized plants containing procyanidins into four groups based on their "procyanidin fingerprint." The configuration of NK 300 tannins was determined to be associated with (+)-catechin, procyanidin B-1, and associated oligomeric procyanidins. Inspection of molecular models has facilitated the prediction of preferred shapes (Fletcher et al. 1977; Haslam 1977). On this basis, sorghum procyanidin would be a right-hand helical structure. The central core would be composed of rings A and B of (-)-epicatechin with ring C projecting laterally from this core and with a terminal (+)-catechin unit.

Molecular-Size Influences

The size of condensed tannin molecules has long been known to influence their protein-binding activity (Goldstein and Swain 1963; Ribereau-Gayon 1972). One tannin molecule binds with two or more peptide groups, primarily through hydrogen bonding, and thus forms cross-links between protein chains (Calderon et al. 1968) and causes precipitation. Flavan monomers and dimers are apparently too small, whereas highly polymerized tannins are either too insoluble or too bulky to fit between suitably oriented protein molecules. Significant protein-binding activity is usually associated with oligomers containing 3–10 monomeric residues (Goldstein and Swain 1963; Roux 1972). Larger-sized molecules are referred to as polymers. Up to a certain molecular weight, as the molecular size of the procyanidin oligomer increases so does astringency. Molecular size also influences the ability of tannins to tan hides (White 1956), to inhibit enzymes (Byrde et al. 1960), and to reduce the nutritive value of foods (Martin-Tanguy et al. 1977).

Changes in Sorghum Tannin during Ripening

Goldstein and Swain (1963) have studied the changes in tannins of several fruits during ripening. They concluded that loss of astringency upon ripening is probably related to increased tannin polymerization with resulting reduced solubility and protein-binding capacity. Similar changes in ripening sorghum seed have been widely referred to, until recently, as a "disappearance of tannin." Results of sorghum maturation studies indicate a general increase in tannin content through the immature stages and then, with few exceptions, a decrease or plateau for the fully ripened grain (Tipton et al. 1970; Johari et al. 1977; Davis and Hoseney 1979b; Price et al. 1979; Bullard et al. 1979b). In our studies of eight BR sorghum varieties in four stages of maturity (Bullard et al. 1979b) we observed an increase in vanillin, Folin-Denis, and leucoanthocyanin values (Table 1) through the mid- to late-dough stages and a subsequent decline as maturity was reached. Additional tests, which measure protein-tannin interactions directly (α-amylase inhibition and protein binding) or indirectly (preference testing with *Quelea*), revealed much the same pattern. The *Quelea* preference means were consistent with those of other chemical and biochemical assays. Individual t-tests indicated significant reductions in *Quelea* preference for the immature stages but analysis of variance did not show any overall differences. A combination of animal variations in behavioural tests and the fact that five of the eight varieties were high in tannin (discussed later) probably accounts for the reduced sensitivity (Bullard et al. 1979b).

The overall decrease (though not significant at $p = 0.05$) in vanillin/Folin (V/FD) and vanillin/

Table 1. Means for chemical and biochemical assays of tannin values in eight developing and ripening bird-resistant (BR) sorghum varieties.[1]

Stage	Chemical assays[2]					Biochemical assays[3]		
	Vanillin-H_2SO_4 (CE)	Folin-Denis (CE)	Leucoantho-cyanin (Cyn E)	V/FO (%)	V/LA (%)	Protein binding (TAE)	α-amylase inhibition (%)	*Quelea* preference (%)[4]
Milk	0.18*a*	0.46*a*	0.30*a*	39.1*a*	60.0*a*	1.09*a*	19.9*ab*	38.2*a*
Light dough	0.31*b*	0.76*b*	0.66*b*	41.8*a*	46.9*a*	1.37*ab*	23.7*ab*	34.2*a*
Firm dough	0.43*b*	0.85*b*	0.74*b*	50.6*a*	58.1*a*	1.47*ab*	24.2*a*	30.8*a*
Mature	0.16*a*	0.47*a*	0.45*ab*	34.0*a*	35.5*a*	1.61*b*	11.8*b*	37.5*a*

[1]Aliquots of combined acetone, methanol, and 90% methanol extracts of samples from eight BR sorghum varieties in four stages of maturity. Discussed in detail in Bullard et al. (1979c).

[2]Chemical assays of extract aliquots conducted by the respective procedures of Hillis and Swain (1959) expressed in catechin equivalents (CE) for Vanillin-H_2SO_4 and Folin-Denis assays and cyanidin equivalents (Cyn E) for leucoanthocyanin assay.

[3]Biochemical assays of extract aliquots conducted by following procedures: α-amylase inhibition in percent by Barnes and Blakely (1974); protein binding in tannic acid equivalents (TAE) by Hagerman and Butler (1978); *Quelea* preference in percent by Bullard and Shumake (1979).

[4]Preference response of *Quelea quelea* when given a choice between millet having surface-coated extract and control millet: percent preference = ((test food consumed)/(test + control food consumed)) × 100.

NOTE: Values followed by the same letter are not significantly different at the 0.05 level of Duncan's Multiple Range Test.

leucoanthocyanin (V/LA) from the firm dough to the mature stage, indicates that polyphenols in sorghums grow larger in molecular size after ripening begins (Table 1). The V/FD ratio has been used by Goldstein and Swain (1963) as an indicator of polyphenol molecule size. Vanillin reagent reacts in an approximately stoichiometric manner with unsubstituted phloroglucinol groups in tannins, and Folin-Denis reagent reacts nonstoichiometrically with hydroxyl groups, therefore the ratio can be used as an indicator of molecular size. However, the V/LA value is often used because both reagents react with flavanols, whereas the Folin-Denis reagent also reacts with nonflavan phenols. We favour the V/FD because of the problems with the leucoanthocyanin analysis (Lewak 1968) and because only sorghum in which the polyphenols are predominantly flavanols are involved (Bullard et al. 1979b).

The increasing V/FD values from the milk stage through the firm dough stage are consistent with ongoing synthesis of vanillin-reactive molecules from smaller phenols. The Folin-Denis reagent would be reactive with all intermediates, whereas vanillin could not react until phloroglucinol-containing molecules have been synthesized.

This pattern in developing and ripening sorghum seeds is consistent with the findings of Haslam (1977). He observed that as chlorophyll forms in the seed coat of sorghum, procyanidins are rapidly synthesized initially and then remain approximately constant until luteolinidin pigmentation occurs. Subsequently, a decrease in flavan-3-ol and associated co-metabolites is seen as relatively insoluble higher molecular weight polyphenols are formed.

Group Chemical Classification of Sorghums

However, the foregoing description of the properties of sorghum tannins is not a complete picture. Cummings and Axtel (1973) and more recently Price et al. (1978) have categorized sorghums into three groups (I, II, or III) on the basis of differences between vanillin and modified vanillin assays. The two assays produce similar values for tannins in group III sorghums whereas the modified vanillin values are much higher than the unmodified vanillin for those varieties included in group II. Group II sorghums are of special interest because, in spite of the presence of a coloured testa and of tannin, they do not appear nutritionally inferior to low-tannin varieties (Cummings and Axtel 1973; Oswalt 1975; Hartigan 1979).

We have conducted *Quelea* preference tests and modified vanillin analyses and obtained Sephadex LH-20 gel permeation chromatographic profiles on 15 BR varieties with different genetic backgrounds (Bullard et al. 1979a). There was a significant negative correlation ($r = -0.88$) between food preference and CE by the modified vanillin assay of Maxson and Rooney (1972) as modified by Price et al. (1978) and between preference and peak area for tannins ($r = -0.66$) in the chromatographic profiles. However, there were definite inconsistencies in some varieties until they were grouped. With few exceptions, the intervarietal properties of group II and group III sorghums were similar whereas intragroup properties were different. Varieties in groups I and II had very similar chemical properties, elicited similar preference response in *Quelea*, and both were different from group III. Chromato-

Table 2. Comparative chemical and biochemical assays of tannin activity in bird-resistant (BR) sorghum by group.[1]

Variety	Group	Protein binding (TAE)[2]	Vanillin (CE)[3]	Folin-Denis (CE)[4]	Leuco-anthocyanin (Cyn E)[5]	V/FD (%)	V/LA (%)
Bravis-BR-ATG-051	I	1.62*fg*	0.55*h*	0.40*h*	0.04*i*	137.5*f*	1375.0*a*
IS 3063c	I	1.58*g*	0.45*h*	0.53*g*	0.18*j*	84.9*f*	250.0*b*
TAM 2566	II	1.82*ef*	0.05*i*	0.23*j*	0.06*hi*	21.7*gh*	83.3*cd*
IS 2266c	II	1.80*efg*	0.05*i*	0.33*hi*	0.11*h*	15.2*h*	45.4*d*
IS 2319	II	1.99*de*	0.05*i*	0.23*j*	0.06*hi*	21.7*gh*	83.0*cd*
Hegari	II	1.78*efg*	0.10*i*	0.24*ij*	0.06*hi*	41.7*g*	166.6*bc*
AKS-614	III	2.72*ab*	2.65*c*	1.35*b*	0.93*b*	196.3*cd*	284.9*b*
RA-Bird-Go-68	III	2.90*a*	4.05*a*	1.55*a*	1.12*a*	261.3*a*	361.6*b*
AR 3005	III	2.89*a*	3.50*b*	1.50*a*	1.12*a*	233.3*ab*	312.5*b*
Funks G516 BR	III	2.51*bc*	2.25*de*	1.24*bc*	0.80*c*	181.4*d*	281.2*b*
BR-54	III	2.58*bc*	2.45*cd*	1.13*e*	0.72*d*	216.8*bc*	340.3*b*
IS 2403c	III	2.30*cd*	1.50*g*	0.88*f*	0.50*f*	170.4*de*	300.0*b*
ROKY 78	III	2.48*bc*	1.80*fg*	1.23*cd*	0.87*c*	146.3*e*	206.9*bc*
IS 2801c	III	2.30*cd*	1.70*fg*	0.89*f*	0.66*e*	191.0*cd*	257.6*b*
WGF	III	2.60*ab*	1.95*ef*	1.15*de*	0.73*d*	169.6*de*	267.1*b*

[1]Extract obtained by shaking ground sample with absolute methanol for 20 min at 30°C (Price et al. 1978). Respective analyses conducted on duplicate extracts.
[2]Protein binding assay conducted by method of Hagerman and Butler (1978).
[3]Vanillin assay conducted by method of Price et al. (1978).
[4]Folin-Denis and leucoanthocyanin assays conducted by method of Hillis and Swain (1959).
[5]Values followed by the same letter are not significantly different at the 0.05 level of Duncan's Multiple Range Test.

graphic profiles indicated that very little tannin was extracted from group II sorghums in comparison to group III while variable quantities of nontannin pigments were present in both.

In another experiment, samples were extracted for 20 min at 30°C with methanol (Price et al. 1978) and vanillin, Folin-Denis, leucoanthocyanin, and protein precipitation assays were conducted on each extract. In Table 2, we again observe large differences between group II and III varieties, while values within the groups were similar. Since absolute methanol was used in a brief extraction, we would expect polymer extraction to be minimal and the V/LA value would decrease for the oligomers extracted (Goldstein and Swain 1963). The smaller V/FD and V/LA ratios indicate that the mean size of oligomers in the extracts of group II was larger than that for group III. Therefore, size of the extracted as well as the unextracted tannins (see modified vanillin analysis obtained under same conditions in Table 3) is one explanation for the differences in properties of groups II and III.

When results of the evaluation of ripening sorghum discussed earlier (Bullard et al. 1979b) were arranged by group, the values were similar for both group II and group III varieties during the immature stages. However, with the exception of the protein-binding values, they were different for the mature grain. Although these particular group II varieties had slightly lower chemical and biochemical activity than group III during the immature stages, this pattern is an encouraging indication that intermediate-sized oligomers with protein-binding qualities are being formed during seed development. Thus, there is a good possibility that sorghum can be developed that is both bird resistant and of acceptable nutritional quality by careful selection of genomes within group II varieties.

Possible Explanations of Group II and III Differences

An attractive hypothesis for explaining the differences between groups II and III is that the biosynthetic process of group II tannin polymers goes to a higher overall degree of completion during ripening than does that of group III. Group III would still contain a substantial proportion of protein-binding oligomers present in the mature grain, while group II would not. However, in testing this hypothesis we found that molecular size was not the only factor involved. The 15 mature grain samples discussed earlier, after vanillin analyses were reextracted for 3 h at 40°C with 50% methanol, which is designed to extract large molecules (Goldstein and Swain 1963), and then reacted with vanillin reagent

Table 3. Determining if the influence of HCl in the modified vanillin analysis affects only tannin solubility.

Variety	Group	Modified vanillin (CE)[1]	Vanillin (CE)[1]	2nd extraction vanillin (CE)[2]	Total vanillin (CE)[3]
Bravis-BR-ATG-051	I	0.10	0.55	0.005	0.56
IS 3063c	I	0.70	0.45	0.080	0.53
TAM 2566	II	0.55	0.05	0.005	0.06
IS 2266	II	0.30	0.05	0.065	0.12
IS 2319	II	0.50	0.05	0.070	0.12
Hegari	II	0.70	0.10	0.025	0.12
AKS-614	III	1.80	2.65	0.045	2.70
RA-Bird-Go 68	III	3.25	4.05	0.045	4.09
AR 3005	III	2.15	3.50	0.085	3.58
Funks G516 BR	III	1.95	2.25	0.110	2.36
BR-54	III	2.05	2.45	0.070	2.52
IS 2403c	III	1.85	1.50	0.190	1.69
ROKY 78	III	1.85	1.80	0.140	1.94
IS 2801	III	0.90	1.70	0.075	1.78
WGF	III	1.60	1.95	0.035	1.98

[1]Extract obtained by shaking ground sample with absolute methanol (vanillin) or 1% HCl in methanol (modified vanillin) for 20 min at 30 °C (Price et al. 1978).
[2]Extract obtained by shaking previously extracted ground sample an additional 3 h at 40 °C with 50% methanol which is designed to extract difficultly soluble tannin polymers (Goldstein and Swain, 1963).
[3]Combined vanillin and 2nd extraction vanillin values.

(Table 3). When the two CE values from the two vanillin analyses for group II varieties were compared and analyzed by a paired t-test, they were still significantly lower than the modified vanillin CE values ($p = 0.01$). Similar comparisons with group III varieties yielded CE values that were significantly higher ($p = 0.007$) than the modified vanillin CE values. There were no differences in the two CE values for group I.

This indicates that the addition of 1% HCl in methanol for modified vanillin extracts affected more than just molecular size influences on tannin solubility. Important differences in starch (Davis and Hoseney 1979a), protein (Tamir and Alumot 1969), and cell wall polysaccharides (Goldstein and Swain 1963) may differentially influence binding with fragments of ground grain (Blessin et al. 1963). For example, Loomis and Battaile (1966) report that in addition to hydrogen bonding, phenols combine irreversibly with proteins by oxidation followed by covalent condensations. Price et al. (1979) have advanced a hypothesis that tannin vesicles become disrupted near maturity in group II sorghum grains causing enzyme polymerization and irreversible binding to some components of the pericarp.

Thus, there is a possibility that some or all of these factors account for differences in tannin properties between groups II and III. From our experience, another likely source of differences is based on the proanthocyanidin composition of the tannins. This, in turn, most likely affects molecular shape, which, according to Quesnel (1968), is at least as important as size. For example, Bate-Smith (1975) observed that prodelphinidins, which are second to procyanidins in frequency of distribution among plants (Thompson et al. 1972), are much more astringent than procyanidins. The chemical nature of proanthocyanidin monomers could also influence ultimate molecular size by affecting the rate and degree of tannin biosynthesis either enzymatically by polyphenol oxidases (Weinges 1964) or thermodynamically through the biogenetic process proposed by Haslam (1974). We have seen some indication that the peak of tannin activity occurs earlier in group II than in group III varieties.

Without exception, all of the group III methanol extracts of mature grain developed a scarlet colour during leucoanthocyanin analyses while those for group II were light brown. Acidic methanol (1% HCl) extracts of group II sorghums varied in colour from the same method of analysis; two were scarlet, two reddish-brown, and one brown. This is a good indication that not all sorghum tannins are exclusively procyanidin in composition and that substantial variations in composition can occur. In addition there are variations in testa colour (York 1976) which further strengthen this possibility.

Researchers at the University of Arkansas Agronomy Department, which supplied most of the BR varieties for the tests described, observed that testas of group II varieties AR 3009 and TAM 2566 had a different colour response in bleach tests than any of the group III varieties examined. They have also observed microscopic differences between the testas of varieties from groups II and III (York, personal communication).

Future Prospects for Sorghum Development

There are many unknown properties which differentiate the group II and III sorghums. There are several literature references to varieties which have resisted bird depredation in the field and demonstrated good nutritional quality in feeding or digestion trials (Thayer et al. 1957; Damron et al. 1968; Harris et al. 1970; Mabbayad and Tipton 1975; Nelson et al. 1975; Oswalt 1975; Tipton et al. 1975). Similarly, no or very little enzyme-inhibition activity has been observed in varieties with a pigmented testa and/or sizeable levels of tannins (Maxson et al. 1973; Daiber 1975). One of the group II varieties tested in this laboratory (TAM 2566) has performed well under heavy bird depredation in Arkansas during the past five years (York, personal communication). Others tested did not withstand severe depredation in Puerto Rico this past winter (1978/79). A better understanding of the differences in polyphenolic composition and the biosynthetic process by which the active polyphenols are produced in sorghum will provide direction in the genetic selection of optimal characteristics for both the desireable bird-resistant qualities in immature seeds and nutritional qualities in the mature grain product.

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Polyphenols in *Pennisetum* Millet[1]

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Polyphenols are responsible for an undesirable gray discoloration in millet flour that creates a problem of consumer acceptance. Bleaching these pigments in dilute acid markedly improved the aesthetic quality of the grains. The major pH-sensitive pigments were identified as glucosylvitexin, glucosylorientin, and vitexin in the relative proportion of 29:11:4. These compounds are concentrated in the peripheral layers of the kernel. Nutritional studies have shown that these phenolic compounds are not as noxious as tannins present in the testa layer of some sorghum varieties.

Pearl millet *(Pennisetum typhoides)* plays a very important role in the agriculture of many developing countries. It is widely grown in Africa and Asia as a food grain. Although yields of millet are generally low in comparison to those of other grains, millet will yield better than most cereals under adverse heat and limited rainfall conditions.

Considerable confusion exists in the millet literature with regard to millet species. Table 1 lists common names and scientific names of the most well-known species (Rachie 1974). In some places the name *Pennisetum typhoides* has been changed to *Pennisetum americanum* and this has added to the confusion. This presentation will deal exclusively with the *Pennisetum* millet that is most commonly referred to as pearl or bulrush millet.

The Millet Colour Problem

Our interest in millet polyphenols is based mainly on aesthetic rather than nutritional considerations. Polyphenols in the grain are responsible for a gray pigmentation which some populations in the world find objectionable.

This problem was discovered by workers who began to mechanize grain processing in Maiduguri, Nigeria. The objective of this IDRC-supported work was to replace the traditional mortar and pestle method of dehulling and grinding grain with mechanical dry-milling equipment (Anonymous 1976). A mill was established at Maiduguri incorporating a grain cleaner and destoner, a dehuller (Reichert and Youngs 1976), a hammermill, sifter, and a packaging facility. The grains which were initially processed included maize, sorghum, and millet. Consumer acceptance was excellent for maize and sorghum flour. However, millet flour met with so much consumer resistance that the mill eventually had to stop processing millet. The gray colour of the millet flour produced by this mill was responsible for the consumer resistance, because the traditionally prepared product was creamy-white in colour.

A survey (Rolston 1975) of millet processing in this area revealed that villagers did not process their millet by a completely dry process. Following mortar and pestle dehulling, the grains were often soaked overnight in water containing tamarind pods or sour milk. This additional processing had a remarkable whitening effect; the original gray colour disappeared completely.

The objective of this paper is to review progress towards understanding this problem, and to suggest some possible solutions. Some of this work has been fully described in Cereal Chemistry (Reichert 1979; Reichert and Youngs 1979). Results are also reported from a preliminary rat-feeding experiment in which millet fractions following dehulling and containing different concentrations of polyphenols were compared with similar fractions obtained by dehulling high- and low-tannin sorghum grains.

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Table 1. Common and scientific names of millet species (Rachie 1974).

	Common name	Scientific name
1.	Bajra, pearl millet, bulrush millet, cattail millet, dukhn, cumbo, sajja, dark millet, candle millet	*Pennisetum typhoides* Stapf. and Hubb.→ *Pennisetum americanum* (L.) K. Schum.
2.	Italian or foxtail millet	*Setaria italica* Beauv.
3.	Proso or common millet	*Panicum miliaceum* Linn.
4.	Little millet	*Panicum miliare* Lam.
5.	Ragi or finger millet	*Eleusine coracana* Gaertn.
6.	Koda or ditch millet	*Paspalum scrobiculatum* Linn.
7.	Japanese barnyard millet	*Echinochloa frumentacea* (Roxb.) Link.
8.	Jungle rice or shama millet	*Echinochloa colona* (L.) Link.
9.	Australian millet	*Echinochloa decompositum*
10.	Browntop millet	*Brachiaria ramosa* (Linn.) Stapf.
11.	Teff	*Eragrostis tef* (Zucc.) Trotter
12.	Fonio or hungry rice	*Digitaria iburua* Stapf.
13.	Fonio or hungry rice	*Digitaria exilis* Stapf.
14.	Adlay or Job's tears	*Coix lachryma-jobi* Linn.

Material and Methods

Grain Samples and Processing

The 16 millet varieties analyzed for polyphenols were the same as those previously used (Reichert and Youngs 1979). The millet, low-tannin sorghum (LTS), and high-tannin sorghum (HTS) used in the nutritional trial were obtained from Senegal in 1977. These varieties were Souna III, Sorgho CE90, and Sorgho X3055, respectively. A long-season variety of millet was obtained from Maiduguri, Nigeria in 1974. All other work reported here was done using a commercial, short-season variety obtained from Maiduguri, Nigeria in 1975.

Soaking treatments in acid, water, or sour milk were described previously (Reichert and Youngs 1979).

Small samples of millet were dehulled in a Strong-Scott barley pearler and the degree of dehulling was determined by the amount of fines passing through a 20-mesh screen (Reichert and Youngs 1976).

Fractionation of grains for the rat-feeding trial was accomplished using a Hill grain thresher at 770 rpm and a cleaning fan speed of 1800 rpm (Reichert and Youngs 1976). Grains were ground in a coffee grinder prior to preparation of diets.

Polyphenol Analysis

Tannins were measured by the vanillin-HCl method using blanks as described by Price et al. (1978).

C-glycosylflavones and alkali-labile ferulic acid (ALFA) which were previously identified and quantified in millet (Reichert 1979) by paper chromatographic and spectrophotometric methods were quantified in 16 millet varieties. Samples for spectrophotometric determination were prepared as previously described. The concentration of C-glycosylflavones was determined in the methanol extract at alkaline pH by application of the formula C=(0.667)(A/23205) (Reichert 1977). The term A is the absorbance at 387 nm; 0.667 is the relative contribution of C-glycosylflavones to the absorbance (from paper chromatography); 23205 is the extinction coefficient; and C is the concentration in moles/litre. Moles/litre were converted to mg/100 g millet flour based on the molecular weight of glucosylvitexin (594.5). The concentration of ALFA was determined in the ether extract of the base hydrolyzate of methanol-extracted millet flour (Reichert 1979). The formula C=(0.898) (A/19863) was used to calculate the concentration, where A is the absorbance at 317 nm; 19863 is the extinction coefficient; 0.898 represents the relative contribution of ferulic acid to the absorbance (from paper chromatography); and C is the concentration in moles/litre (Reichert 1977).

Flour-Reflectance Measurements

Reflectance properties of dry flours and flour pastes were measured in terms of absorbance units. Measurements were made using a Hitachi Perkin-Elmer spectrophotometer equipped with a diffuse reflectance attachment (Reichert 1977 and 1979).

To determine reflectance characteristics of the 16 millet varieties at alkaline pH the millet flours were diluted with wheat starch. Twelve grams of wheat starch was thoroughly mixed with 0.5 g of millet flour. Ten millilitres of 0.1 *N* NaOH was added and then mixed for 3 min before a reflectance measurement at 375 nm was made on the slurry.

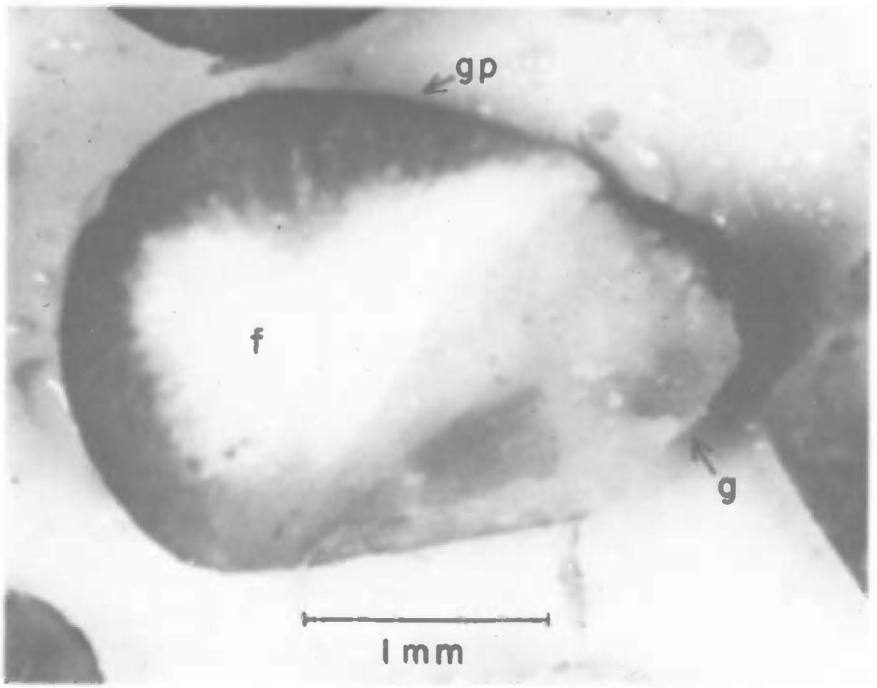

Fig. 1. Photograph of whole millet grain showing the location of gray pigmentation (gp), germ (g), and floury endosperm (f).

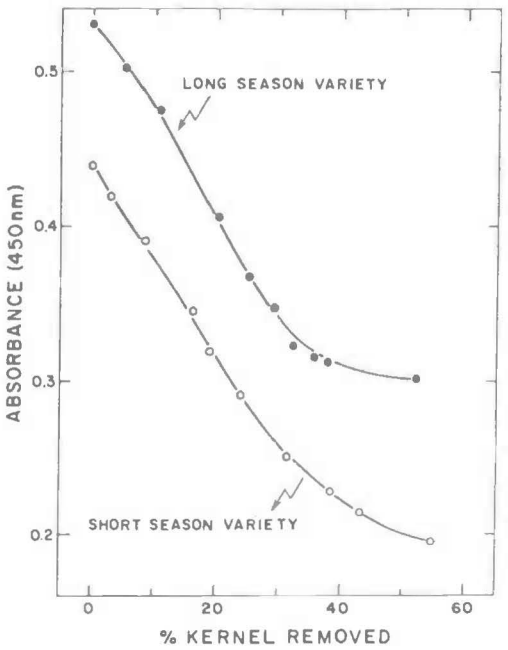

Fig. 2. The absorbance (450 nm) of dry flours prepared by progressively dehulling long- and short-season millet varieties.

Nutrition Study

Fractions from the abrasive dehulling of millet, HTS, and LTS were evaluated for nutritional quality using weanling male Wistar-strain rats. These fractions were incorporated at 60% of the feeding ration. The protein content of all diets was equalized at 23% by the addition of casein. The crude fibre content (based on crude fibre content plus added cellulose) was 6% in each diet. Methionine was supplemented to meet the requirement of the growing rat as specified by Warner and Breuer (1972). Six rats per ration were fed and watered ad libitum for 28 days. Feces collected during the last two weeks were analyzed for chromic oxide (Bolin and Lockhart 1960).

Results and Discussion

Distribution of the Gray Pigment in Millet

The gray pigment was predominantly located in the peripheral area of the seed (Fig. 1). The central portion of the seed was very white in comparison. A considerable improvement in flour colour was made by simply pearling the grain in a barley pearler (Fig. 2), in which process

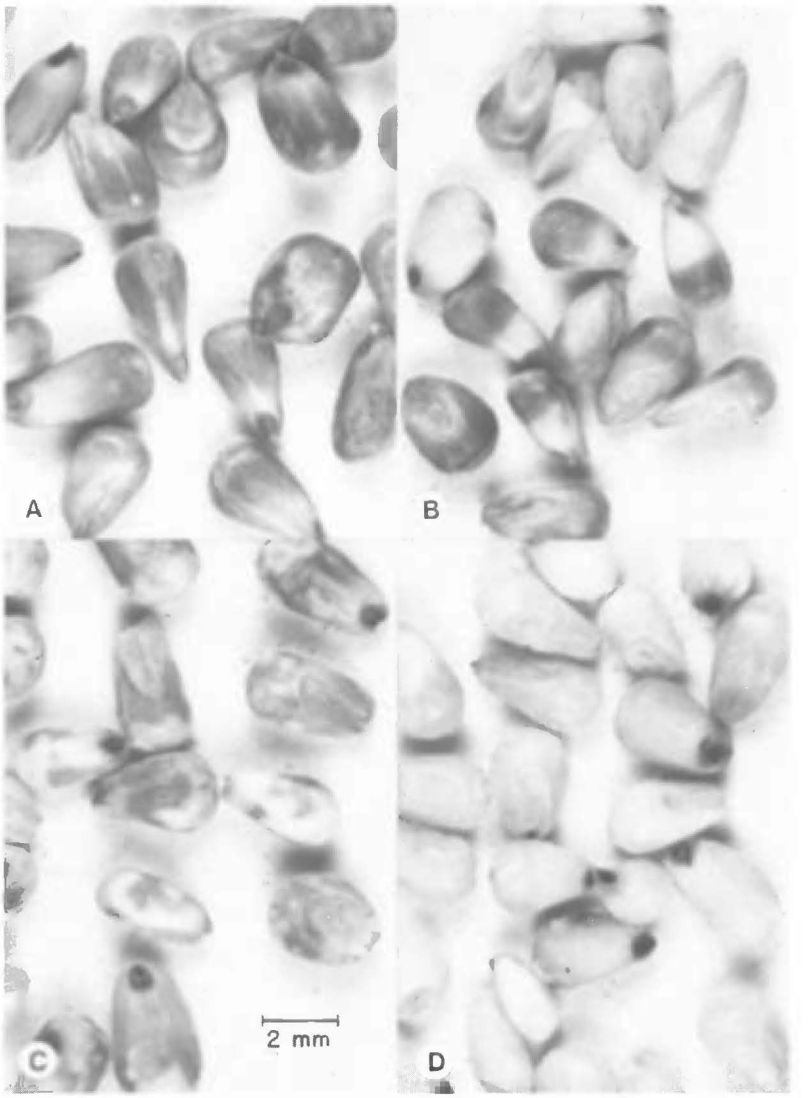

Fig. 3. Photographs of whole and dehulled millet grains demonstrating the influence of acid bleaching on exterior colour intensity. A. untreated whole millet; B. whole millet soaked in 0.2 N HCl for 12 h; C. scarified millet grain (2.4% removed) soaked in 0.2 N HCl for 20 min; D. same as C but soaked 3 h.

the outer layers are abraded off by a carborundum stone. The losses incurred in pearling a sample to an acceptable degree of whiteness were, however, in excess of 45%.

Acid Bleaching of Millet

The traditional bleaching treatments of millet grain, which involve soaking grains in sour milk or tamarind pod solutions, lower the pH to 4.5–5.0. It was found that soaking the seeds in H_2O caused a slight reduction in colour, but that soaking in an acidic medium such as 0.2 *N* HCl caused the same dramatic colour change as did the traditional method.

Fig. 3 illustrates the dramatic improvement in grain colour achieved by soaking whole or dehulled grains in 0.2 *N* HCl. Whole millet grain

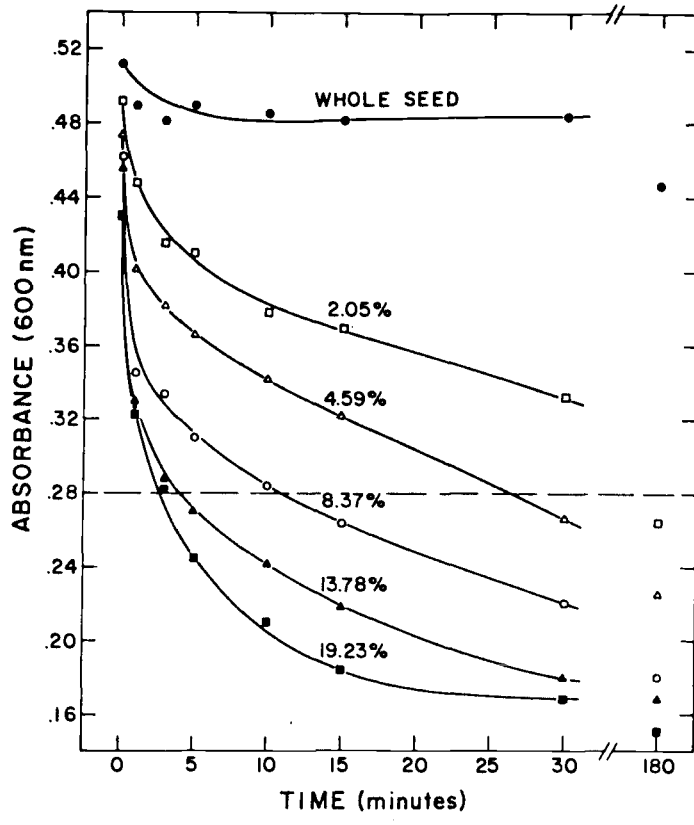

Fig. 4. Absorbance of flour pastes from millet which was abrasively dehulled to various degrees (% kernel removed) and soaked in 0.2 N HCl for various time intervals (time, min). The broken line represents the absorbance of a sample that was traditionally dehulled (mortar and pestle) and soaked overnight in a solution containing sour milk.

absorbed the acid slowly through the embryo (Fig. 3B). The progression of the acid was easy to follow because of the distinct colour boundary observed in many seeds. Some seeds required as long as 48 h for the acid to migrate to the opposite end. However, scarification of millet grain in the barley pearler allowed rapid absorption of the acid through all areas where the husk had been broken (Fig. 3C).

The relation between the degree of scarification and the rate of acid absorption into millet is illustrated in Fig. 4. Whole millet grain showed very little colour change during the 3-hour soaking in 0.2 *N* HCl, whereas seeds that had 19.2% kernel removed with the barley pearler whitened very rapidly. The traditionally prepared product, which was dehulled in a mortar and pestle and soaked in a solution containing sour milk, gave an absorbance value of 0.28. To achieve this degree of whiteness, typical combinations of degree of pearling and soaking time are: 4.6% kernel removed and soaked 26 min; 8.4% kernel removed and soaked 11 min; 13.8% kernel removed and soaked 4 min; and 19.2% kernel removed and soaked 3 min.

On treatment with acid, some varieties of millet lighten in colour to a greater degree than others (Fig. 5). All varieties were soaked in 0.1 *N* HCl for 48 h and then air-dried. The treatment was judged to be excellent where the treated grain was white in colour. These groups included PHB-14, Serere 2A-9, Maiwa composite, white, and deep slate. Two varieties responded very little to acid bleaching and these were the purple and deep purple varieties. All other grains were yellow in colour after treatment. This indicates that the gray or yellow-gray pigment is easily bleached, whereas the yellow or brown pigment is not.

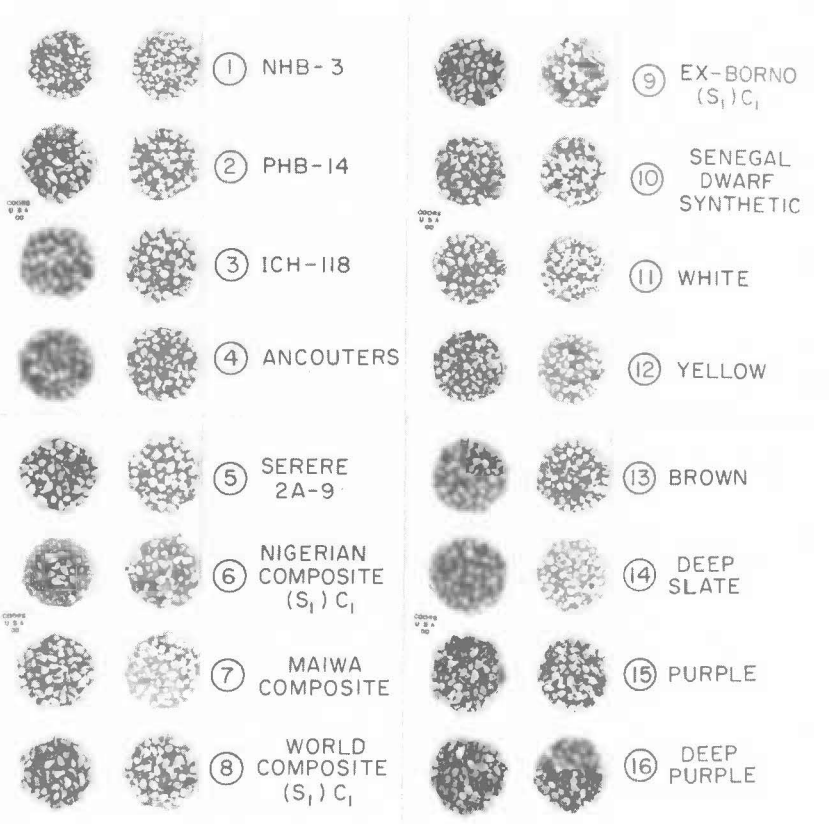

Fig. 5. A comparison of pericarp colours of 16 varieties of millet before (left) and after (right) soaking in 0.1 N HCl.

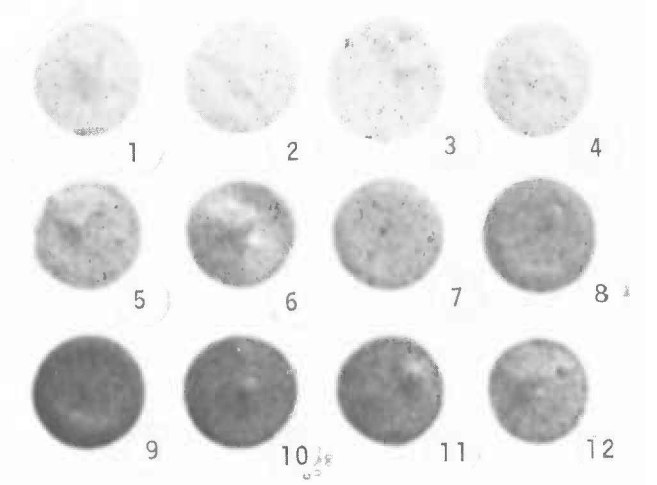

Fig. 6. Millet flour pastes at pH 1–12. Pastes from pH 1 to 4 were creamy white, from pH 5 to 7 they were gray, and from pH 8 to 12 they were bright yellow-green in colour.

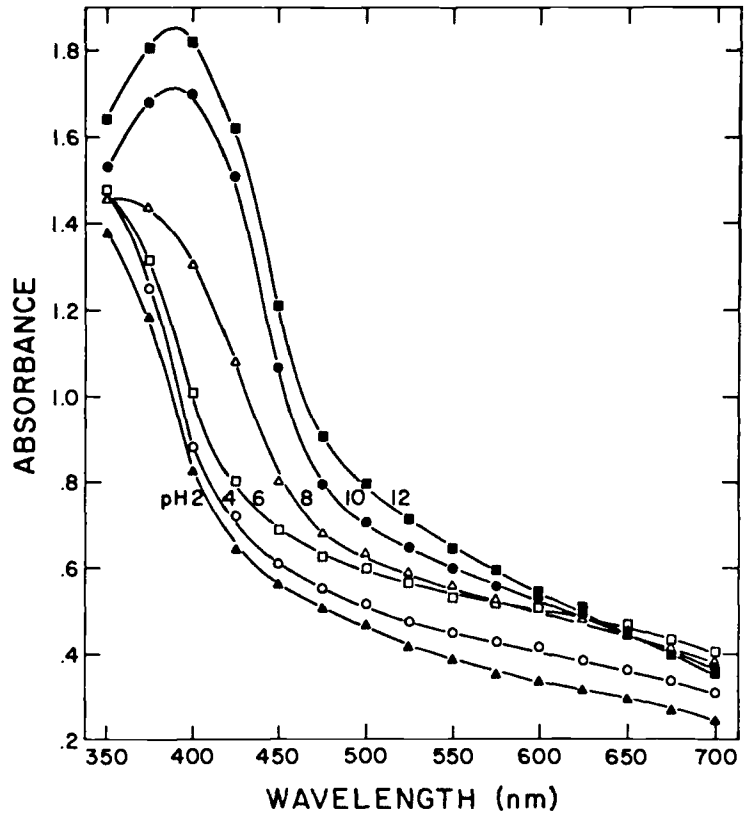

Fig. 7. Influence of pH (2–12) on absorbance of millet flour pastes at wavelengths of 350–700 nm.

pH-Sensitive Pigments in Millet

As a function of pH, millet flour pastes exhibited a range of colours (Fig. 6). Flour pastes from pH 1–4 were creamy-white, from pH 5–7 they were gray, and from pH 8–12 they were bright yellow-green in colour. Reflectance spectra of these same pastes showed the appearance of an absorption maximum at approximately 390 nm which was evident only under alkaline conditions (Fig. 7).

The pigments responsible for the yellow-green colour of millet flour pastes at alkaline pH were methanol soluble. The natural gray pigmentation in the peripheral area of the seed was also methanol soluble. This was demonstrated by simply cracking the grain to expose the endosperm and stirring the broken kernels in methanol for a few hours. After this treatment the gray pigment had disappeared completely.

Examination of paper chromatograms of methanol extracts of millet flour did not reveal the presence of any gray pigment. However, the pigments responsible for the yellow-green colour of millet paste at alkaline pH were easily recognizable. The structures of the pigments were elucidated by UV spectrophotometric techniques using diagnostic reagents as described by Mabry et al. (1970). Sugar groups were identified by paper chromatography following acid hydrolysis (Pridham 1956). The structure of these compounds and their relative proportions in millet are shown in Fig. 8. Glucosylvitexin, glucosylorientin, and vitexin are from a class of flavonoids known as C-glycosylflavones. These compounds are characterized by having a sugar bound via a carbon-carbon bond to the flavonoid nucleus. This sugar group is not hydrolyzable by normal acid or enzymatic hydrolysis procedures.

Glucosylvitexin was the major pH-sensitive pigment in millet, and the pure compound showed an absorption maximum at alkaline pH of 395 nm. Similarly, glucosylorientin and vitexin showed absorption maxima at 405 nm and 395 nm, respectively, under alkaline conditions. This illustrated that these compounds were likely

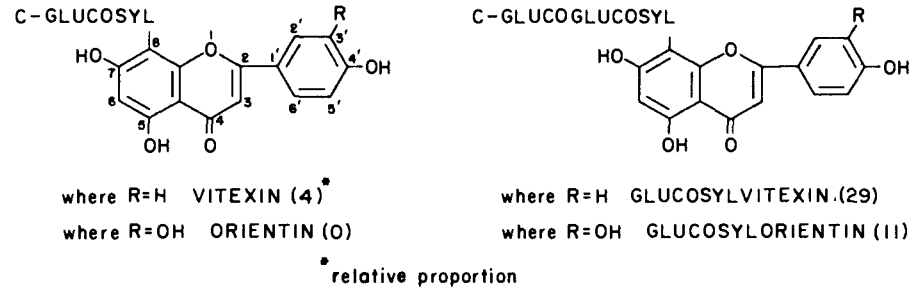

Fig. 8. Structures and relative proportions of the major C-glycosylflavones in millet.

responsible for the reflectance maximum of the millet flour paste (alkaline pH) observed at 390 nm.

Methanol-extracted millet flour also contained a substantial quantity of ferulic acid which was liberated by alkaline hydrolysis. Some of the pH sensitivity which methanol-extracted millet flour still exhibits could be due to ferulic acid esterified to glucose, quinic acid, other sugars, or an amino acid.

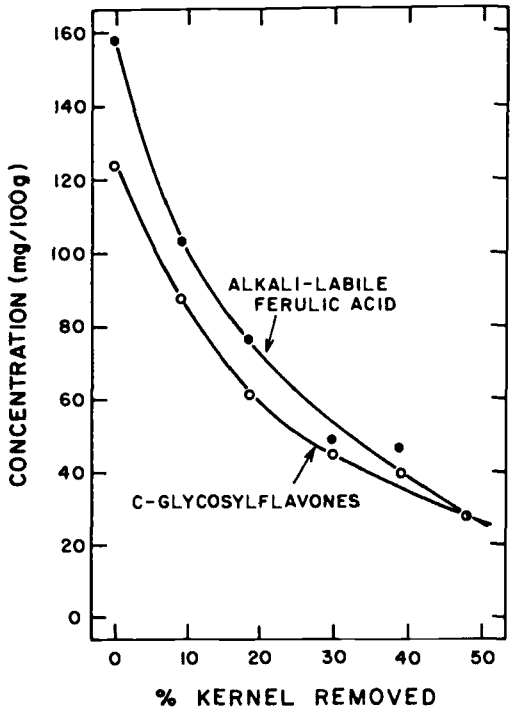

Fig. 9. Effect of the degree of kernel removal on the concentration of C-glycosylflavones and ALFA.

The concentrations of C-glycosylflavones and ALFA in whole and dehulled millet are illustrated in Fig. 9. Whole millet contained 124 mg/100 g of C-glycosylflavones and 158 mg/100 g of ALFA. When the grain was dehulled, concentrations of these phenolics decreased markedly. The correlation coefficient between C-glycosylflavones and dry flour absorbance (450 nm) of pearled grains was 0.994. Similarly the correlation coefficient between ALFA and dry flour absorbance (450 nm) was 0.991. This indicated that the decrease in colour of millet flour and the decrease in the polyphenol content were well correlated following abrasive dehulling of this millet.

To further investigate the relationship between colour and polyphenol concentration, a number of grain types were analyzed for reflectance properties and polyphenol content (Table 2). The reflectance measurement of the flour paste (natural pH of 6.3) at 450 nm is a measure of whiteness. The reflectance measurement of the flour paste (pH 11.2) at 375 nm is a measure of the degree of yellowness or yellow-green pigmentation in the paste. The pigmentation of whole millet flour pastes at alkaline pH was so intense that dilution of the paste with wheat starch was necessary to obtain reproducible and reliable reflectance values.

Considerable variation existed in the grain colour and whiteness of the flour among the various types analyzed (Table 2). This character of grain colour needs to be considered by plant breeders in the future selection of food-grade millet varieties. The C-glycosylflavone content varied from 87 to 259 mg/100 g, while the ALFA content varied from 127 to 241 mg/100 g. The values reported in the table are only approximate, as a paper chromatographic analysis was not done on all samples. The degree of yellowness of the flour paste at alkaline pH also varied considerably as shown in the last column. These pastes ranged in colour from very pale yellow for

Table 2. Colour, polyphenol content, and reflectance properties of bulrush millet varieties.

Variety	Grain colour	ALFA[1] (mg/100g)	C-GF[2] (mg/100g)	Abs (450nm) of flour paste, pH 6.3	Abs (375nm) of flour paste, pH 11.2[3]
NHB-3	gray-yellow	175	151	0.676	0.590
PHB-14	gray	190	127	0.681	0.573
ICH-118	gray-light brown	161	87	0.667	0.534
Ancouters	brown-yellow	185	259	0.813	0.654
Serere 2A-9	gray-green	220	134	0.645	0.559
Nigerian composite $(S_1)C_1$	gray-light brown	144	111	0.639	0.532
Maiwa composite	yellow-gray	133	124	0.519	0.527
World composite $(S_1)C_1$	gray-light brown	153	113	0.674	0.540
Ex-Borno $(S_1)C_1$	gray-brown	156	124	0.670	0.573
Senegal dwarf synthetic	yellow-green	241	119	0.705	0.580
White	yellow	137	107	0.564	0.549
Yellow	yellow-green	210	172	0.707	0.630
Brown	light brown	175	139	0.775	0.637
Deep slate	gray	127	113	0.673	0.566
Purple	brown	188	158	0.780	0.610
Deep purple	dark brown	199	130	0.792	0.582

[1]Alkali-labile ferulic acid.
[2]C-glycosylflavones.
[3]4% Flour in wheat starch adjusted to pH 11.2.

ICH-118 to a much more intense yellow-green for Ancouters.

Correlation coefficients between the concentrations of polyphenols and colour are illustrated in Table 3. As was expected, the C-glycosylflavones are responsible for much of the colour of the millet flour paste at alkaline pH. It appears, however, that the whiteness of the natural flour is not related, or only very weakly related, to the total C-glycosylflavone or ALFA content.

The gray pigmentation appears to be a result of a complex interaction rather than the result of the presence of a single compound. Indeed, the in vivo colour of a compound may be due to many factors which could include chelation of the phenolic in vivo with Cu, Fe, Al, or other metal ions; copigmentation effects which enhance colours; or a pH effect which changes the degree of ionization of the phenolic (Singleton 1972).

Table 3. Correlation of polyphenol content and millet colour.

	Flour paste absorbance	
	450nm,pH 6.3	375nm,pH 11.2
C-glycosylflavones	0.57	0.80
ALFA	0.51	0.48

Effect of Millet Polyphenols on Nutritional Quality

A preliminary experiment was conducted to determine if the dehulled millet fractions, containing different amounts of polyphenols, had any adverse effect on rats fed a diet containing 23% protein (supplemented with casein) and all other nutrients necessary for a complete ration. Fractions were prepared by abrasive dehulling in a Hill grain thresher, which gradually removes the peripheral layers of the grain. A high-tannin (HTS) and low-tannin sorghum (LTS) were similarly processed. All fractions were fed at a 60% level in the diet.

Proximate analysis of these fractions (Table 4) demonstrated a marked concentration of the fat, protein, fibre, and ash into the hull fractions. The most marked fractionation of protein occurred in millet, indicating that the protein is not as uniformly distributed throughout the seed as in sorghum.

Fractionation of the tannins, as determined by the vanillin-HCl method, occurred during processing of the HTS grain. This grain was extremely difficult to dehull because of its soft endosperm structure. Millet or LTS contained no measurable level of tannin. In fact, all varieties of millet studied so far have appeared free from tannins. The polyphenols reported in this paper (C-glycosylflavones and ALFA), however, were

Table 4. Yields, proximate analysis, and tannin analysis of grain fractions (% dry basis).

Fraction	Yield (%)	Crude fat (%)	Protein (%)	Ash (%)	Crude fibre (%)	Tannin (%)
LTS	—	2.81	13.28	1.76	2.03	0
Dehulled LTS	85.2	2.05	12.57	1.34	0.78	0
LTS hull	14.8	8.24	14.32	4.18	8.72	0
HTS	—	2.77	11.35	1.61	1.85	3.60
Dehulled HTS	57.8	1.60	12.00	1.02	1.10	1.43
HTS hull	42.2	4.52	11.41	2.47	3.62	5.85
Millet	—	5.21	11.10	1.73	1.92	0
Dehulled millet	86.6	3.86	10.50	1.26	0.77	0
Millet hull	13.4	13.52	18.26	4.56	6.75	0
Wheat flour	72	0.99	12.71	0.43	0.18	0

Table 5. Effect of dehulling on the nutritional quality of millet and high- and low-tannin sorghum.

Fraction	Feed intake (g)	Weight gain (g)	Feed/gain	Dry-matter digestibility (%)	Protein digestibility (%)
LTS	415.9*a*	179.5*a*	2.32*a*	86.8*a*	87.3*a*
Dehulled LTS	420.1*a*	187.6*a*	2.24*a*	88.2*a*	89.9*b*
LTS hull	505.6*b*	182.9*a*	2.78*b*	74.2*b*	81.6*c*
HTS	427.7*a*	176.8*a*	2.42*a*	82.9*a*	79.9*a*
Dehulled HTS	435.7*a*	188.8*a*	2.31*a*	87.6*b*	86.1*b*
HTS hull	503.0*b*	182.2*a*	2.77*b*	75.7*c*	69.5*c*
Millet	423.2*a*	183.5*ab*	2.31*a*	86.1*a*	88.4*a*
Dehulled millet	441.4*a*	195.5*a*	2.26*a*	88.6*b*	90.0*a*
Millet hull	439.8*a*	170.6*b*	2.58*b*	72.6*c*	77.4*b*
Wheat flour	432.3	193.6	2.21	90.4	91.6

NOTE: Each grain was analyzed separately for purposes of statistical evaluation. Values in any one column followed by different letters are significantly different.

present and the dehulled millet seed would be expected to contain approximately 40% less than the whole seed. The concentration of polyphenols in the hull fraction is about 3 times that in the whole grain.

Table 5 shows that in the feeding experiments dehulled millet was not significantly different from whole millet in feed intake, weight gain, feed/gain ratio, or protein digestibility. Values for the LTS fractions were very similar to those of millet fractions. Dehulled LTS was not significantly different from whole LTS in feed intake, weight gain, feed/gain ratio, or dry-matter digestibility. However, protein digestibility was somewhat improved by dehulling. Dehulling HTS caused a marked improvement in dry-matter and protein digestibility.

From this experiment it appears that the millet fractions performed in a similar manner to the LTS fractions in all nutritional variables measured. It appears, therefore, that any problems associated with millet polyphenols are of the same general magnitude as those associated with LTS. Much work needs to be done to clarify the effect of simple nontannin phenolics on nutritional quality.

Conclusions

Polyphenols in millet grain are not as nutritionally adverse as the tannins present in the testa layer of some cultivars of sorghum. However, these polyphenols present an aesthetic problem because of a gray pigmentation in the peripheral areas of the seed. Consumer acceptance of millet flour produced by a dry-milling facility in an African country was considered significantly inferior to the traditionally accepted product, a creamy-white millet flour that villagers produced by soaking dehulled grain in solutions containing sour milk or tamarind pods.

One solution to the problem of consumer acceptance might be for the mill simply to sell dehulled millet grain to consumers who could themselves treat the grain in whichever way they were accustomed. Consumers could return the treated and dried grain to the mill to be ground into flour.

Alternatively, the mill could set up a bleaching plant using a chemical to lower the pH. Lactic acid is responsible for lowering the pH of sour milk. Similarly, tartaric acid is responsible for lowering the pH when tamarind pods are used in the traditional process. Either of these chemicals or simply dilute HCl could be used to bleach millet. Such a plant would require a drying facility to reduce the moisture content of treated grains.

A third solution is for plant breeders to breed for varieties with less colour. This is a long-term approach, but may be the most satisfactory.

The Nutritional Role of Polyphenols in Beans

Ricardo Bressani[1] and Luiz G. Elías[1]

The nutritional role of polyphenols in food legumes remains unclear. White, black, and red *Phaseolus vulgaris* contain 0.34–0.42, 0.57–1.15, and 0.95–1.29% polyphenols as tannic acid mainly in testa; protein quality is higher for white, black, and red, respectively. Cooking destroys known antiphysiological factors, but not tannins, which are partially removed with cooking liquor. Bean protein quality is lower when assayed with cooking liquor, for red and black, but not white. Polyphenols decrease protein digestibility in animals and humans, probably by making protein partially unavailable or by inhibiting digestive enzymes and increasing fecal nitrogen.

Legume foods provide the supplementary protein to diets based on either cereal grains or starchy food, commonly consumed in developing countries. Thus, increasing attention is being given to agricultural programs aimed at increasing yield and, hopefully, availability to consumers.

It is important, however, to consider in such programs the introduction of improved nutritional-quality characteristics as well as acceptable cooking and organoleptic qualities (Hulse et al. 1977).

Much attention has been given in the past to the destruction of the well-known antiphysiological factors in legume foods, such as trypsin inhibitors, by appropriate processing. Likewise some reports have focused on the establishment of nutritional standards (Hulse et al. 1977), which, besides protein and specific essential amino acids, include other characteristics related to acceptability and ease of preparation for consumption. On the other hand, polyphenolic compounds in food legumes have not been as thoroughly investigated, and there are only a few studies in animals which indicate that these compounds affect nutritional quality (Chang and Fuller 1964; Lindgren 1975; Marquardt et al. 1978). However, there is need to know more about their possible role in relation to storage, cooking quality, and nutrient utilization. Legume grain protein has a low digestibility, which has not been adequately explained; furthermore, after long storage legumes become difficult to cook and are, therefore, refused by the consumer. Finally, although mothers in developing countries do not feed whole cooked legume foods to their children because of their undesirable effects, they do often feed cooking broth to 1- to 3-year-old children (Bressani et al. 1973). The question was then raised as to whether polyphenolic compounds in common beans and other legume foods play a role in the constraints indicated above.

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Polyphenol Content in *Phaseolus vulgaris* Strains

Polyphenolic compounds in common beans have been determined either by the method of Folin-Denis (Joslyn 1970) and expressed as tannic acid, or by the vanillin-HCl method of Burns (1971) and expressed as catechin equivalents (CE). Reports on the subject are relatively few, however those available show similar results as shown in Table 1. The materials in the table represent strains of common beans grown in Wisconsin, Puerto Rico, and Guatemala. When the polyphenols were determined by the Folin-Denis method, the values were higher than when expressed as catechin equivalents. Independent of how the results were calculated, however, they indicate differences with respect to seed coat colour. White beans show the lowest amounts of polyphenols, which in general increase in black, red, and bronze varieties. The variability in white-coated beans is relatively small, however it is much greater in materials with black, red, and bronze seed coats. Studies on tannin-content inheritance in common beans have shown that it has a high broad-sense heritability (Ma Yu and Bliss 1978; Ronnenkamp 1977), therefore, low-

Table 1. Polyphenol content in *P. vulgaris* cultivars.

Sample origin	*n*	Testa colour	Catechin equiv. value (mg/g)	Tannic acid (%)
Wisconsin[1]	6	white	2.31 (1.50–3.26)	—
	4	black	6.65 (2.48–18.86)	—
	3	bronze	7.80 (7.10–8.16)	—
Puerto Rico[1]	1	white	2.40	—
	9	black	5.30 (3.40–10.60)	—
	4	red	12.56 (7.18–15.10)	—
Guatemala[2]	31	white	—	0.38 (0.16–0.53)
	249	black	—	1.13 (0.72–1.77)
	39	red	—	1.14 (0.87–1.52)
Guatemala[3]	3	white	0.24 (0.18–0.28)	—
	10	black	1.99 (0.62–5.90)	—
	4	red	6.42 (1.87–10.06)	—
	3	bronze	9.19 (0.38–14.10)	—

[1]Ma Yu and Bliss 1978.
[2]Bressani and Braham 1978.
[3]Linares and de Bosque 1978.

Table 2. Polyphenols in red-coloured beans expressed as tannic acid and as catechin equivalent.

Cultivar No.	Tannic acid (%)	Catechin equiv. value (mg/g)
1	0.91	15.9
2	0.77	6.6
3	0.86	9.8
4	0.77	13.0
5	0.91	25.4
6	0.70	4.0
7	0.82	5.6
8	0.95	21.0
9	0.92	14.8
10	0.89	11.0
11	0.81	10.5
12	0.93	16.2
13	0.95	17.8
$\bar{X}$	0.87	13.2
r	0.65**	

tannin strains in coloured cultivars may be obtained either by selecting among existing pure lines, or by crossing and selecting for appropriate recombinations. The importance of this finding is that since populations have strong colour preferences, it is possible to select coloured seeds with low polyphenol content, meeting at the same time consumer preferences, such as colour and flavour.

With respect to the analytical methodology for polyphenol compounds in grain legumes, there is a high correlation between "tannic acid" (Folin-Denis) and catechin equivalent (vanillin-HCl) (Bressani et al., in preparation) as shown in Table 2. In this case, 13 red-coloured cultivars were analyzed by both methods. It would be desirable, however, to have a more specific methodology and to know exactly the type of phenolic compounds being analyzed.

Tannins in common beans are located in the seed coat of the grain, with low or negligible amounts in the cotyledons. Some results in this respect are shown in Table 3. The values reported were done on cotyledons without seed coat, on the seed coat, or on the whole seed. The results show that the cotyledons contain lower concentrations than the whole seed, while the seed coat is the main source of polyphenolic compounds in common beans, whether expressed as catechin equivalent or tannic acid. According to this, there should be a correlation between percentage seed coat and polyphenol content, and between polyphenol content and seed size. However, the various studies reported did not show such a relationship, indicating that phenolic content is independent of seed size (Ma Yu and Bliss 1978; Elías et al. 1979).

Effects of Processing

Common beans are cooked before consumption to make them soft and to destroy antiphysiological substances. Though the cooking process may vary, it generally follows the sequence of steps shown in Fig. 1. Soaking beans in water for

Table 3. Polyphenol distribution in anatomical fractions of *P. vulgaris*.

Sample origin	Testa colour	Whole seed	Cotyledons	Seed coat
Catechin equiv. value (mg/g)				
Wisconsin[1]	white	2.31	2.17	—
	black	6.65	2.90	—
	bronze	7.80	2.04	—
Puerto Rico[1]	white	2.40	0.44	—
	black	5.30	0.98	—
	red	12.56	1.01	—
Tannic acid (%)				
Guatemala[2]	white	3.85	4.15	1.30
	black	7.95	5.25	42.50
	red	9.30	5.00	38.00

[1]Ma Yu and Bliss 1978.
[2]Elías et al. 1979.

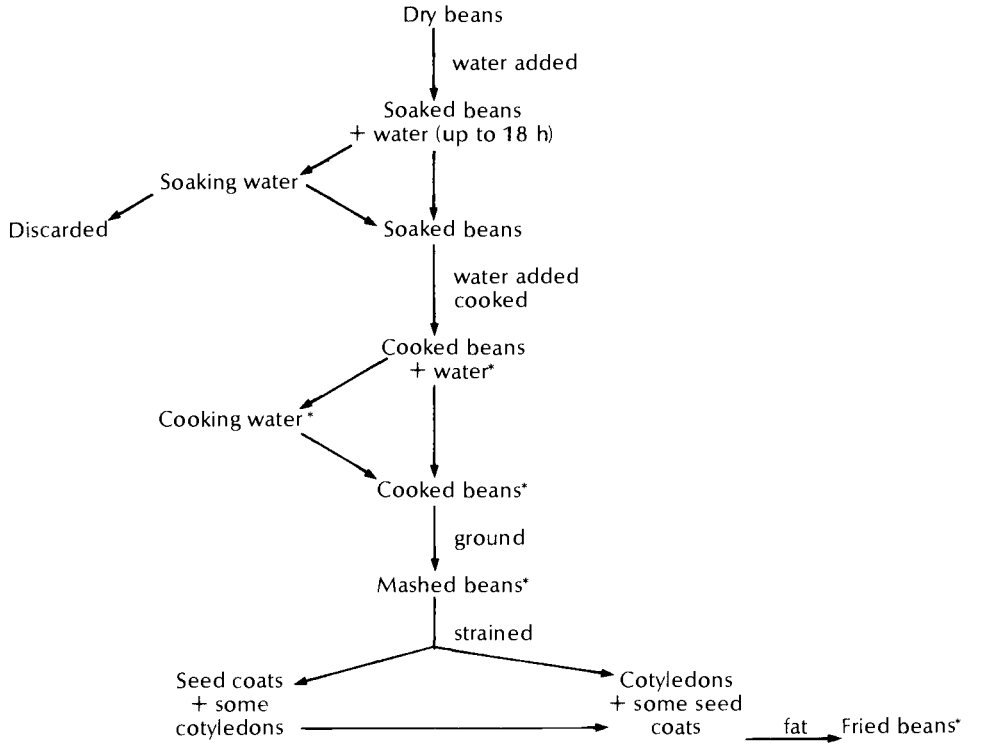

Fig. 1. Processing of beans for consumption in Latin America. (Asterisks indicate fractions and/or products consumed.)

periods up to 18 h is a common practice in Latin America. The soaking water, which contains around 1.3% solids, may or may not be discarded. Cooking is carried out in additional water for periods of up to 4 h at atmospheric pressure, or for about 30 min under 15 pounds (6.8 kg) of pressure. The cooking water may be removed to be used as a soup, particularly for children, or it may be left in with the beans. Cooking water alone usually contains 4–10% solids. Beans may be then crushed and consumed or strained and fried (Bressani et al. 1973).

Several studies have been carried out to learn of the changes which may take place during cooking. Table 4 summarizes results of cooking black, red, and white bean samples. The cooked material was analyzed without removing the cooking water. It can be seen that polyphenols expressed as tannic acid decreased 30–49% on cooking (de España 1977). The degree of change is variable and at present its nature cannot be explained.

Because no physical separation was made, the change may have resulted from the binding of polyphenols with other organic substances, or from alterations in chemical structure of the polyphenols, thus rendering them incapable of giving the chemical colour reaction measured by the Folin-Denis method.

Table 4. Losses of polyphenols during cooking, expressed as tannic acid (de España 1977).

Bean colour	Tannic acid (%)		
	Raw	Cooked	Loss (%)
Black	0.90	0.63	30.0
Red	1.21	0.62	48.8
White	0.40	0.27	32.5

Large amounts of polyphenols may be found in the cooking waters as indicated in Table 5. In these examples, the samples were analyzed raw, and cooked, with and without the cooking broth. The results show lower values in the cooked bean samples analyzed without the cooking broth (Elías et al. 1979; Fukuda Suzuki 1978). The lower section of the table shows the comparatively high amounts found in the cooking broth. Similar studies have been performed, expressing polyphenolics as catechin equivalents, and some results are shown in Table 6. Losses in this case appear to be much higher than when tannic acid is used to indicate polyphenol content (Linares and de Bosque 1979). As indicated, no explanation is available at present to account for the loss. It is possible the polyphenols react with carbohydrate, protein, or other substances which could be measured biologically, or may be partially destroyed.

Table 6. Losses in polyphenols during cooking, expressed as catechin equivalent value, mg/g (Linares and de Bosque 1979).

Bean colour	Raw	Cooked
Black	1.99	0.24
Red	6.42	0.12
White	0.24	0.12
Brown	9.19	0.38

Attempts to obtain some explanation have been made by following the changes which occur during the processing of the raw beans to crushed cooked beans. The results, as shown in Table 7, indicate that although relatively large amounts of polyphenols could be eliminated by discarding washing and cooking waters, the residue, mainly cotyledons, retains large quantities, because of the apparent migration of the tannins from the seed coats to the cotyledons. The actual amounts ingested will thus depend on how beans are processed and consumed.

Nutritional Role

It is recognized that tannins alter the nutritional quality of plant products. This has been very well demonstrated for bird-resistant grain sorghum (Chang and Fuller 1964; Lindgren 1975).

Table 5. Polyphenols in raw and cooked *P. vulgaris* with and without the cooking broth, expressed as tannic acid.

Colour of seed	Raw	Cooked - cooking broth	Cooked + cooking broth	Cooking broth
White[1]	0.35	0.10	0.20	—
Black[1]	0.75	0.34	0.45	—
Red[1]	0.97	0.49	0.45	—
White[2]	0.38	0.20	—	0.78
Black[2]	0.80	0.52	—	0.76
Red[2]	0.93	0.41	—	2.10

[1]Fukuda Suzuki 1978.
[2]Elías et al. 1979.

Table 7. Distribution of polyphenols as tannic acid during cooking of *P. vulgaris*[1], grams.

Bean product	Bean colour		
	Black	White	Red
1) Raw beans	4.50	1.80	7.35
2) Soaking water	0.16	0.05	0.25
3) Soaked beans	3.80	1.94	5.23
4) Soaking water + soaked beans	4.43 (3.96)[2]	2.11 (1.99)	5.78 (5.48)
5) Cooked beans	2.72	1.20	2.75
6) Cooking water	0.86	0.28	0.86
7) Cooked beans + cooking water	3.37 (3.58)[2]	1.48 (1.48)[2]	4.19 (3.61)[2]
8) Mashed beans	3.58	1.48	4.67
9) Strained	(0.73%)[3]	(0.34%)[3]	(0.74%)[3]
10) Seed coat	(1.00%)	(0.28%)	(1.27%)
Loss (1-8) (%)	20.4	17.8	36.5

[1]Values represent absolute amounts from an initial sample of 500 g of beans.
[2]Values in parentheses represent calculated result.
[3]Content in protein.

Table 8. Protein digestibility of beans of different colour.

Bean colour	Tannin (%)[1]	Dig. (%)[1]	Tannin (%)[2]	Dig. (%)[2]
Black	0.63	70.7	0.34	74.6
Red	0.62	68.5	0.49	70.1
White	0.27	75.2	0.10	82.5

[1]de España 1977.
[2]Fukuda Suzuki 1978.

Table 9. Correlation coefficients between polyphenols as catechin equivalent and in vivo protein digestibility (Linares and de Bosque 1979).

All samples	$n = 80$	$r = -0.3955^{**}$
Black	$n = 40$	$r = -0.369$
Red	$n = 12$	$r = -0.1031$
Bronze	$n = 16$	$r = -0.3987$
White	$n = 12$	$r = -0.1264$

Some reports have indicated that tannins in different cultivars of peas, field beans, and other food legumes were responsible for lower digestion coefficients for crude protein in poultry (Lindgren 1975; Marquardt et al. 1978).

Artificial rumen studies have shown that tannin extracts from carobs inhibit cellulolytic and proteolytic activity (Tamir and Alumot 1969). Furthermore, the carob tannins have been shown to be strongly inhibitory of trypsin and amylase and, to a lesser degree, of lipase. These results suggest that the depressing effect of tannins results from their action on digestive enzymes. However, they can react with food proteins, interfering with their digestion by enzymes, and, consequently, lowering amino acid availability (Haslam 1974).

Some information on the nutritional role of polyphenols in *Phaseolus vulgaris* has already been obtained. Table 8 describes results from two independent studies showing a relation between polyphenols as tannic acid and protein digestibility (de España 1977; Fukuda Suzuki 1978). In both studies white-coated cultivars showed the highest digestibility and the lowest tannic acid content. Furthermore, red-coloured beans in both studies showed the lowest protein digestibility with the highest tannic acid content. It should be indicated that in both studies the cooking broth was separated from the cooked cotyledons. Larger numbers of samples have been studied recently and Table 9 presents the correlations found between phenolic content and in vivo protein digestibility. These are significantly negative for all samples taken together as well as for black-coated beans. All other correlations are negative although not statistically significant (Linares and de Bosque 1979). As shown previously, the broth contains relatively large amounts of polyphenols and, if included with the cotyledons, it may decrease digestibility further (see Table 10). The addition of the cooking broth to both black- and red-coloured beans decreased

Table 10. Effect of cooking broth addition on protein digestibility of beans of different colour (Elías et al. 1979).

Sample	Protein digestibility (%)		
	White	Red	Black
Cooked beans	81.3 ± 1.8	78.7 ± 2.8	77.9 ± 2.5
Cooked beans + broth	81.4 ± 1.5	70.4 ± 4.8	75.0 ± 3.5

Table 11. Effect of cooking broth addition on average weight gain and protein efficiency ratio (PER) of various food legumes (Elías et al. 1979).

Identification	Ave. wt. gain		PER	
	+ cooking broth	- cooking broth	+ cooking broth	- cooking broth
Sensuntepeque (black) *(P. vulgaris)*	6	29	0.20	0.88
S-184-N (black) *(P. vulgaris)*	-1	29	—	0.95
Red-70 (red) *(P. vulgaris)*	8	10	0.31	0.48
27-R (red) *(P. vulgaris)*	21	27	0.61	0.86
Cowpea *(V. sinensis)*	32	52	1.04	1.24
Soybean *(G. max)*	94	113	1.69	1.80
Pigeon pea *(C. cajan)*	36	51	0.98	1.46

protein digestibility, however such an effect was not obtained with white-coloured beans. It should be indicated, however, that differences in digestibility may not be due to tannins alone. Availability of amino acids may also play a role still to be demonstrated.

The effect of adding the cooking broth is also seen in terms of protein quality as determined by the protein efficiency ratio (PER). Some representative values are shown in Table 11. In this case, the elimination of the broth as part of the bean in the diet resulted in higher PER values. This is clear for *P. vulgaris,* as well as for other food legumes, such as cowpeas, soybeans, and pigeon peas, although not as striking as for *P. vulgaris* (Elías et al. 1976). It is of importance to recognize that the effect may not be entirely due to tannins but may result from interactions of the protein with some unidentified adverse component of the broth.

The results of two studies of interest are shown in Table 12 (de España 1977; Fukuda Suzuki 1978). In the first, there is a direct relationship between polyphenol content and the net protein ratio (NPR) value of the legume protein. The relationship between tannin content and protein quality is also evident when the protein is supplemented with methionine, thus eliminating the possibility of the role this amino acid plays in the protein quality of legume foods. In the second study, the assay utilized was the nitrogen growth index (or NGI, which is determined as the slope of the line relating growth rate to protein intake). The assay is carried out by feeding rats diets with different levels of protein from the same source. As more beans form part of the diet, more phenolics are included in it. The results obtained suggest again that coloured beans have a lower protein-quality value than white beans. Additional studies are now under way to ascertain the relationship between polyphenols in beans and protein digestibility.

Studies have also been conducted with adult human subjects (Hernández 1979). Preliminary results from four subjects show that black beans had the lowest protein digestibility, followed by red beans, while both were lower than the digestibility value of cheese protein (see Table 13). These studies are now being expanded to include white beans with and without the cooking broth from

Table 12. Relationship between polyphenol content in beans and their protein quality with and without the addition of methionine to the diet.[1]

	Tannin[2] (%)	- methionine	+ methionine
Net protein ratio (NPR)			
Black[3] $n = 5$	0.47–0.81 (0.63)	2.00	3.18
Red[3] $n = 2$	0.59–0.64 (0.62)	1.96	2.88
White[3] $n = 2$	0.27–0.28 (0.27)	2.38	3.90
Nitrogen growth index (NGI)			
Black[4] $n = 3$	0.29–0.38 (0.34)	1.81	—
Red[4] $n = 2$	0.48–0.50 (0.49)	2.09	—
White[4] $n = 2$	0.08–0.11 (0.10)	2.56	—

[1]Both studies conducted without adding the cooking broth.
[2]In cooked sample.
[3]de España 1977.
[4]Fukuda Suzuki 1978.

Table 13. Protein digestibility in humans fed bean protein with different content of polyphenols, expressed as tannic acid (Hernåndez 1979).

Protein source	Nitrogen balance (mg/kg/day)	Digestibility	
		Apparent (%)	True (%)
Red beans	46.6 ± 6.5	55.7 ± 4.7	78.9 ± 4.4
Black beans	46.4 ± 2.8	49.6 ± 2.4	72.8 ± 3.1
Cheese	72.0 ± 3.4	76.2 ± 1.4	98.3 ± 1.6

either red or black beans. In order to find out whether or not tannins are influencing the results, regression equations were calculated, between catechin intake and fecal nitrogen. Fig. 2 shows the results of two regressions. The first, calculated with fecal N excretions from a diet without beans or tannins, and the second, in which this point was not included. There is a statistically significant correlation in the first case. However, in the second case the correlation is not statistically significant. It is believed that there is need to increase the number of observations to be able to be conclusive on this particular point.

In summary, it may be concluded that tannins affect the utilization of protein in beans through increasing fecal N output, as made evident in rat studies. Trials with human subjects showed a similar trend, although the effect was less marked than in rats.

Based on the observations reported in this paper and on those from the literature, it may be suggested that the polyphenol compounds in common beans decrease protein digestibility either by inhibiting digestive enzymes or by reacting with protein, reducing amino acid availability, or both. The decrease in protein digestibility, in turn, reduces absorption of amino acids which could explain the lower protein-quality value observed. In case phenolic compounds are absorbed, they will have to be eliminated, either as glucuronate derivatives or sulfates; in the latter case, sulfur amino acid needs will increase, thus decreasing the quality of the protein for growth purposes. All these effects are, however, relatively small.

With respect to the significance of the polyphenols present in beans and their effect in nutrient utilization, the problem should be analyzed from two points of view. One is the nutritional effect per se and the second, bean acceptability. As for the nutritional effects, these are evident, but relatively small. They would probably be smaller considering that beans are only one component of the diet, thus the effects will be diluted or minimized, and certainly, very difficult to measure.

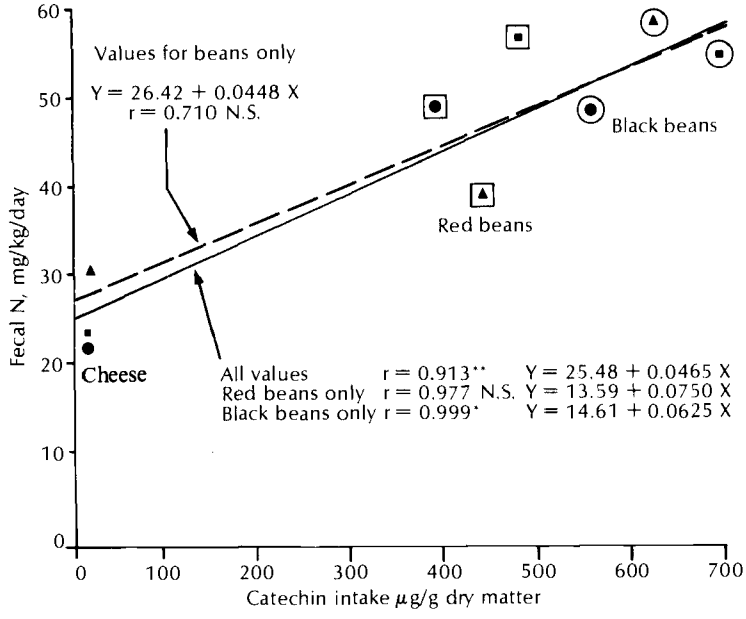

Fig. 2. Relationship between catechin intake and fecal N output in humans (Hernández 1979).

Regarding the problem of bean acceptability, one aspect is clear, at least in Latin America, coloured beans are greatly preferred to white-coated beans, by all populations. Furthermore, the cooking liquors must be thick and dark and are preferred for young children. The question could be asked if acceptability is based only on colour of the seed coat or also on the small amounts of polyphenolic compounds present. It is of interest to point out in this respect that preference varies even within the same colour. Therefore, it would be of interest to find out if phenolic compounds in common beans are related to acceptability and to increase our knowledge of their nutritional role, before genetic selection is undertaken by legume breeders to eliminate them.

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